CHARACTERIZATION OF THE ANTI-INFLAMMATORY EFFECTS OF THE MOLLUSCUM CONTAGIOSUM MC159 PROTEIN

BY

CRYSTAL MARIE HARMON RANDALL

DISSERTATION

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Doctoral Committee:

Associate Professor Joanna Shisler, Chair
Associate Professor Brenda Wilson
Professor Steven Blanke
Associate Professor Richard Tapping

Abstract

The Molluscum Contagiosum Virus (MCV) is a dermotropic poxvirus that strictly infects humans. MCV infections produce umbilicated lesions that can persist for six to nine months in children and young adults. Currently there is no cure for MCV infections. Recent evidence suggests that the pro-inflammatory Tumor Necrosis Factor-α (TNF-α) and anti-viral interferon- β (IFN-β) cytokines are highly expressed in MCV lesions and surrounding tissue. Despite the expression of pro-inflammatory factors, MCV infections cause little inflammation. Not surprisingly, MCV encodes immunomodulatory proteins such as the MC159 protein to combat the effects of TNF-α and IFN-β. The work presented here details MC159 mediated inhibition of these cytokines through the NF-κB and IRF-3 transcription factors.

TNF-α is a critical initiator of the pro-inflammatory response during infection. Previous studies found that MC159 is a potent inhibitor of the TNF-α pathway by blocking activation of the NF-κB pro-inflammatory transcription factor. Mutational analysis revealed that the RxDL motif within the N-terminal DED of MC159 is required for inhibition of NF-κB. Additionally, my results show that MC159 inhibits TNF-α, PMA and MyD88 induced NF-κB activity, suggesting MC159 targets a step in the pathway shared by these inducers. I hypothesized that MC159 targeted the IκB kinase (IKK) complex to inhibit NF-κB activation. To this end, I found that MC159 co-immunoprecipitated with the IKKγ subunit of the IKK complex, suggesting that MC159 targets the IKK complex to inhibit NF-κB activation.

Interferon beta (IFN-β) initiates the primary anti-viral response during infection. IFN-β synthesis is triggered by viral infection when host cellular sensors detect byproducts of viral propagation (e.g. dsRNA). Activation of these sensors initiates a signaling cascade that results in the phosphorylation and subsequent activation of the TBK-1 kinase that in turn phosphorylates the Interferon Regulatory Factor-3 (IRF-3) transcription factor. I hypothesized that MC159 inhibits IFN-β protein synthesis. In support of this hypothesis, I found that MC159 inhibits IFN- β production in an NF-κB-independent manner. In addition, my results show MC159 inhibits phosphorylation of TBK-1 and IRF-3. Taken together, these data suggest that MC159 inhibits IFN-β activation by acting on the TBK-1 kinase complex. Mutational analysis revealed that either Death Effector Domain (DED), a protein:protein interaction domain, of the MC159 protein was sufficient for inhibition. Finally, I show homologs of MC159 including MCV MC160 and cellular $FLIP_L$, inhibit IFN-β expression, suggesting DEDs are important for regulation of IFN-β responses.

To Jason, Claire and William

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Chapter 1: Introduction to thesis

1) Poxvirus overview

The *Poxviridae* family is an extensive group of viruses with a broad host range that includes both vertebrates and invertebrates. Poxviruses are characterized by a large, double-stranded DNA genome of 130-300 kbp, which encodes nearly 200 proteins. Poxviruses are unique from other viruses with DNA genomes, because poxviruses replicate and transcribe their genome exclusively in the cytoplasm of their host cell. To perform this task, poxviruses encode their own DNA replication and transcription machinery. Poxviruses encode a diverse set of proteins that modulate host-pathogen interactions [43, 57]. The study of poxviruses has led to significant advancements in the fields of virology, cellular biology, and immunology. The focus of my thesis is to identify how the MCV MC159 protein modulates host cell responses against infection. Specifically, I identified how the MC159 protein inhibits the activation of the anti-viral NF-κB and IRF-3 transcription factors. Before discussing MC159, it is necessary to understand the properties of poxviruses. This section will cover poxvirus structure and morphology, classification, and replication cycle.

Classification.

Members of the family *Poxviridae* can be organized into two distinct subfamilies: those belonging to *Entomopoxvirinae*, which infect insects, and those belonging to *Chordopoxvirinae*, which infect vertebrates. Members of the subfamily *Chordopoxvirinae* are the most extensively studied and can be further divided into 8

genera: *Orthopoxvirus*, *Parapoxvirus*, *Avipoxvirus*, *Capripoxvirus*, *Lepripoxvirus*, *Suipoxvirus*, *Molluscipoxvirus*, and *Yatapoxvirus*. Although there is a wide range of poxviruses that infect vertebrates, only two of the genera have biological implications for humans, *Orthopoxvirus* and *Molluscipoxvirus.* Of all the viruses belonging to these two genera, only the variola virus (VARV), an *Orthopoxvirus,* and the molluscum contagiosum virus (MCV), a *Molluscipoxvirus,* strictly infect humans. For this reason, I am interested in studying MCV. However, other *Orthopoxviruses* such as cowpox and monkeypox can cause zoonotic disease in humans. Members of the *Orthopoxvirus* genera have long been used as model systems to study poxviral diseases, including vaccinia virus (VV), which was used as a vaccine against variola, and the ecrtromelia virus that infects mice [57].

As of 2006, 110 poxvirus genomes are sequenced. Sequence analysis revealed that there are 49 genes that are shared among all poxvirus genomes [43]. Most of the conserved genes are located at the centers of each genome, and are involved in essential processes such as viral replication, transcription, and virion assembly. In contrast the terminal ends of poxvirus genomes vary greatly between genera. These regions contain genes whose products encode viral immune evasion proteins and host-range proteins [43, 57]. The differences observed between genomes is likely reflective of the different host-range and tissue tropism for each virus.

Structure and morphology.

Regardless of the differences in genes, all poxviruses have a characteristic large barrel-shaped virion that measures 200 nm high and 300 nm long, which can be visualized using a light microscope. Analysis of pox virions using transmission electron microscopy revealed that mature viral particles are wrapped in either a single or double membrane and display a dumbbell shaped core encapsulating the genome, flanked by two lateral bodies [57].

Poxvirus replication cycle.

The poxvirus life cycle has been well characterized using Vaccinia Virus (VV) as a model for all poxviruses (Figure 1.1). Although the Molluscum Contagiosum Virus (MCV) cannot be grown in cell culture the life cycle is predicted to be similar to VV. This section will review viral attachment, entry, genome expression, replication, viral assembly, and egress.

Attachment and entry.

VV provides an excellent model for poxvirus attachment and entry (Figure 1.1). However, unlike VV, MCV has a narrow host range only infecting human keratinocytes. Therefore MCV may have a slightly different mechanism of attachment and entry.

A poxvirus virion must attach to and enter a host cell to begin a productive infection cycle. The process by which a poxvirus attaches and enters a host cell remains unclear. No single receptor has been determined to be necessary for poxvirus attachments. Due to the broad range of host and cell types that poxviruses infect, it is

thought that the mechanism for attachment must involve factors that are ubiquitous [57]. There is evidence that initial attachment is mediated by glycosaminoglycans on the host cells [11]. Mature VV virions contain attachment proteins D8 and A27/H3 that have been shown to bind either chondroitin sulfate (D8) or heparin sulfate (A27/H3) [16, 37, 38, 47, 87]. However, neither protein is essential for entry. Additionally, excess heparin can only partially block virus entry, and only in certain cell types [11].

Although the mechanism of poxvirus attachment remains unclear, poxvirus entry has been intensely studied using VV as a model for poxvirus infection. To date, two models for VV entry have been described. The first is that VV can enter host cells via macropinocytosis [52, 53]. This occurs when VV particles associate with the cell membrane, inducing entry via a low-pH-dependent endosomal pathway [71, 81, 82]. In the second method, VV virions enter host cells via viral and cellular membrane fusion facilitated at cholesterol-rich regions of the host cell membrane called lipid rafts [17]. A highly conserved multi-protein entry complex composed of eight viral proteins is necessary for fusion and viral entry into host cells [57]. Recent studies suggest that the mode of entry varies based on the virus and cell type.

Transcription, translation, and replication of viral genomes.

The mechanism by which poxviruses express and replicate their genomes has been intensely studied. The transcription, translation, and replication machinery is highly conserved amongst poxviruses, and VV is used as a model to study genome replication and expression [7].

Unlike other DNA viruses, poxviruses replicate exclusively in the cytoplasm of the host cell (Figure 1.1). To achieve this, each virion contains transcription machinery packaged in its core, allowing for viral gene expression to begin shortly after entering the cell. Each virion contains a viral RNA polymerase, vaccinia early transcription factors (VETF), and RNA polymerase associate factor (RAP94) [57].

Poxvirus gene expression occurs in a temporal cascade starting with early genes and followed by intermediate and late genes [1, 7, 57]. Recent studies show that early genes are expressed at 20 minutes post infection and at greater levels than the other gene groups [1].

Early genes encode for enzymes involved in DNA synthesis, intermediate gene transcription factors, and host-evasion genes. Early genes have promoter sequences that are A/T rich and are specifically recognized by early transcription factors. Early gene transcripts are terminated by viral termination factors at the UUUUUUNU termination sequence [74, 75]. Thus, early viral mRNAs all have homogenous ends unlike late viral transcripts. Upon termination of early gene transcription, the viral core becomes disrupted. This disruption process contributes to transcription termination via dispersion of the transcription machinery [57]. Two viral mRNA decapping proteins, D9 and D10, can also mediate termination of early transcription. This results in the increased degradation of viral transcripts [64, 65].

Replication of the viral genome is initiated by disruption of the viral core approximately 1-2 hours post infection. The current model for VV replication is a rolling hairpin-strand displacement. In this model, a nick is created at one or both ends of the viral genome, providing a 3'OH for the viral replication machinery to initiate new strand

synthesis. The hairpin structure of the genome permits the DNA to fold back on itself, allowing the entire genome to be copied. However, this process results in concatamer of the newly synthesized and parent genome, which must be resolved for replication to be complete [57].

Once the genome is replicated, transcription of the intermediate genes begins. Intermediate gene transcription peaks around 2 hours post infection. Intermediate genes are the smallest group of genes and are transcribed for the shortest amount of time. The majority of intermediate genes are transcription factors needed for late-gene expression. Intermediate gene transcription peaks around 2 hours post infection [57, 64].

Finally, late-gene transcription begins when enough late-transcription factors have been expressed. Late genes include structural proteins that make up the virus particle and transcription machinery and any other proteins that are packaged in the viral core. Late viral transcripts have been shown to form dsRNA late during infection. The formation of these dsRNAs is likely due to heterogeneity of the late transcripts, which results from read through of the termination sequences [57].

Virion assembly and viral egress.

The final stages of the poxvirus life cycle are virion assembly and viral egress (Figure 1.1). The site of virion assembly in host cells is called a viral factory. Viral factories can be visualized using electron microscopy, and appear as darker dense regions within an infected cell. Within these factories, viral particles are assembled as a result of proteolytic processing of the structural proteins and packaging of the genome. An assembled viral particle within the cell is called a mature virion (MV) or intracellular

mature virion (IMV) [7, 57]. Utilizing the host's microtubules, the MV leaves the viral factories and move toward the cell surface. In the process, the MV acquires a double membrane from the trans-golgi apparatus or endosomes. Once wrapped in a membrane, the MV is called an enveloped virion (EV). For egress to occur, the outer membrane of the viral particle fuses with the cell membrane. Then the viral particles can be disseminated to continue another infection cycle. One mode of dissemination is for the virus to detach from the host cell and become extracellular enveloped viruses (EEV), allowing spread to nearby or distant cells. In a second mechanism, cell-associated EVs are shuttled by actin to neighboring cells [57].

In general, though diverse, the majority of poxviruses share the same replication cycle. Further research is needed to elucidate the fine differences in the replication cycle between MCV and the orthopoxviruses.

2) Molluscum contagiosum virus

MCV causes a self-limiting infection that can persist in healthy individuals for months. MCV infections are common among children and immunocompromised adults. My thesis focuses on the immune evasion mechanisms of the MC159 protein. To better understand the role of MC159 in disease, this section will examine MCV pathogenesis.

MCV pathogenesis.

MCV is a dermotropic poxvirus that solely infects humans. It is the only poxvirus other than the variola virus, the causative agent of small pox, with this narrow host range [10, 57]. However, due to an extensive vaccination campaign, variola virus was

eradicated. MCV infections occur in people of all age groups, but are especially common in children, sexually active young adults, and immunocompromised individuals [6, 84]. Currently, there is no cure for Molluscum Contagiosum (MC) infections, and treatment strategies are limited.

The most interesting property of MCV is that it can only replicate in keratinocytes [84]. MC presents with benign umbilicated neoplasms in the epidermal skin layer. MC is often a self-limiting infection. However, MC infections can persist for six months to five years [84]. MC can be transmitted on surfaces or via skin-to-skin contact. For this reason, MC infections are often sexually transmitted in adults and are present in the groin area. MCV infections account for one percent of all diagnosed dermatologic conditions [84]. In addition, MCV infections are present in five to eight percent of human immunodeficiency virus (HIV) patients. Immunocompromised patients, such as those with HIV, experience much larger lesions (giant MC) and a prolonged infection that persists indefinitely [8].

Even though the MCV host range is limited to humans recent reports have described an MCV-like disease in animals including, donkeys, chickens, sparrows, pigeons, chimpanzees, kangaroos, dogs and horses [23]. *In situ* hybridization analysis suggests considerable homology between human and equine MCV [79]. Similar to human MCV, all attempts to culture these viruses has been unsuccessful. In addition, no animal MCV infection has been successfully experimentally transmitted from one animal to another. Further research is necessary to understand how these MCV and MCV-like viruses are related, and whether their host-ranges overlap.

Yet another striking difference between smallpox and MC is that MC has low inflammation. Although MCV infections are associated with little inflammation, innate pro-

inflammatory pathways are critical for the resolution of MC. Recent evidence suggests that Tumor Necrosis Factor-alpha (TNF-α) is crucial for host defenses during MCV infections. First, TNF-α is highly expressed in MCV lesions and surrounding tissue [42]. Secondly, patients on anti-TNF therapy have increased susceptibility to MCV infection [18].

There are three subtypes of MCV, (MCV-1, MCV-2, MCV-3), with MCV-1 being the most common [41]. Approximately 58% to 77% of MC infected individuals produce antibodies against MCV, suggesting that B cells are important for controlling MCV infections [41, 88]. However, it has been suggested that MC infections are primarily cleared by cell-mediated responses because many individuals with persistent MCV infections have high antibody titers, and antibody production occurs months after initial infection [41]. Although MC antibodies can be detected in 6% to 23% percent of the general population, it is hard to estimate prevalence because many cases remain subclinical [41, 88].

MCV genome.

The MCV genome was sequenced in 1996 [69]. Sequence analysis revealed that MCV encodes approximately 182 proteins, 105 of which are shared with VV [69, 70]. Not surprisingly, the most conserved genes (i) localize to the center of the genome, and (ii) encode proteins that are essential for structure, replication, and transcription. Genes unique to MCV were located at the ends of its genome, and these genes likely encode proteins that function as host-range and immunomodulatory factors. Little is understood concerning how these immunomodulatory factors function. My project focuses on understanding how one such factor, MC159, inhibits NF-κB and IRF-3.

MCV tropism.

Although all attempts to grow MCV outside of the human host have been unsuccessful, the lifecycle of MCV is predicted to be similar to that of VV [21]. However, MCV produces an abortive infection in a human primary fibroblast cell line (MRC-5) [9]. In these cells, MCV expresses early and late proteins but no viable progeny is produced [9]. Since MCV exhibits a strong tissue tropism for the epidermis, it is possible that MCV replication requires factors only present in skin [10]. Another hypothesis is that MCV cannot be grown in tissue culture because it has lost some genes present in other poxviruses that allow for replication in multiple cell types [10, 70]. A third hypothesis is MCV cannot be grown in culture because it lacks the viral kinase B1 encoded by VV [69, 70]. B1 plays a critical role in replication by phosphorylating the cellular protein BAF [62]. Without B1 VV is unable to replicate [62]. A tissue culture model is necessary to fully understand the process of replication during MCV infection. Lack of a tissue culture model presents a challenge for understanding MCV infections. In this study, a surrogate poxvirus (vaccinia) that now encodes MCV proteins was created to gain insight into MCV pathogenesis.

3) Host innate immune signaling

Overview.

The resolution of a virus infection relies on a robust immune response. Innate immunity is critical for the initial detection of and response to viral infection. Nearly all cells are armed with multiple innate immune sensing pathways. Detection of Pathogen

Associated Molecular Patterns (PAMPs), which are often byproducts of viral infection, (dsRNA, dsDNA and glycoproteins), trigger primary immune responses [23]. Myriad cellular sensors such as, Toll-Like Receptors (TLRs) and Retinoic Inducible Gene-I (RIG-I), recognize viral PAMPs. These cellular sensors activate many innate immune signaling pathways such as NF-κB and Interferon-beta (IFN-β) [24]. Once activated, these pathways can initiate multiple outcomes, including inflammation, effectors cell recruitment, induction of an anti-viral state, and apoptosis of the infected cell. All of these pathways will become activated during a poxvirus infection. In fact, poxviruses devote nearly thirty percent of their genomes to counteract the effects of these innate signaling pathways [35, 36]. Here I will review the three pathways the MC159 protein inhibits NF-κB, apoptosis and IFN-β.

NF-κB.

NF-κB is a key initiator of the proinflammatory response, being activated by many cellular pathways such as Tumor Necrosis Factor–Receptor 1 (TNF-R1) and TLRs to regulate over 100 target genes [32, 33, 55]. NF-κB is a cellular transcription factor activated by a diverse set of stimuli, including reactive oxygen species, UV light, cytokines, LPS, dsRNA, and viruses.

There are five known mammalian NF-κB family proteins: p65 (RelA), RelB, c-Rel, p50/p105, and p52/p100 [27]. These proteins act as, homodimers or heterodimers, with each dimer differently contributing to regulation of NF-κB target genes. However, the p65/p50 complex is the most common. NF-κB family members possess RHD (Rel

Homology Domain) motifs, which mediate protein: protein interactions such as dimerization and interaction with IκBs (Inhibitors of NF-κB).

Prior to stimulation, NF-κB resides in the cytoplasm bound by its inhibitor, IκB (Figure 1.2). The IκB family consists of seven proteins, including IκBα, IκBβ, BCL-3, IκBϵ, IκBγ, p100, and p105 [32, 33]. IκB proteins inhibit NF-κB activity by binding its nuclear localization sequence, thus preventing its translocation to the nucleus [33]. IκB proteins each contain five to seven ankyrin repeats, which mediate protein:protein interactions such as IκB-NF-κB interactions.

Upon stimulation of the NF-κB pathway, IκB is phosphorylated by Inhibitor of NF-κB Kinase complex (IKK) [32, 33] (Figure 1.2). The IKK complex is a large multi-protein complex that is composed of three main subunits: two catalytically active kinases, IKKα and IKKβ, and one regulatory subunit IKKγ. This phosphorylation targets IκB for ubiquitination by an SCF ubiquitin ligase and subsequent degradation by the 26S proteosome. The degradation of IκB frees NF-κB and allows it to translocate to the nucleus. The IκB proteins regulate NF-κB activation in a biphasic manner. IκBα binds NF-κB in resting cells, but upon prolonged stimulation, IκBβ is produced and binds NF-κB.

Once in the nucleus, NF-κB binds to a conserved κB sequence (5'-GGGGYNNCCY-3') and initiates transcription of its target genes. Since NF-κB stimulates genes such as cytokines, chemokines, pro-apoptotic factors, and anti-apoptotic factors, NF-κB is a critical player in the up-regulation of immune responses.

Interestingly, much advancement has been made in the immune signaling field through the study of viral proteins. The major targets for viral evasion of NF-κB

activation are the IKK complex and the NF-κB:IκB complex [12, 15, 19, 22, 51, 60, 61, 66]. Chapter 2 of this review will detail my work regarding how the MCV MC159 protein inhibits the NF-κB pathway.

Apoptosis.

Apoptosis or programmed cell death is critical for the execution and clearance of virally infected cells [67]. Therefore, many viruses encode proteins that block the activation apoptosis. Apoptosis is initiated by many different signals including, DNA damage, cellular stress, and infection [67]. One of the best-characterized pro-apoptotic pathways is the Death Receptor, Tumor Necrosis Factor Receptor (TNFR) pathway [31]. TNF-R1 is a potent activator of apoptosis in response to viral infection and is ubiquitously expressed among most cell types [67]. Therefore, TNF-R1 induced apoptosis will be the subject of this section.

Upon stimulation by tumor necrosis factor α (TNF-α), an inflammatory cytokine simulated by NF-κB, TNF-R1, TNFR-2 mediate a diverse range of signaling responses [4]. Both receptors are members of the TNF Receptor superfamily, possessing conserved cysteine-rich extracellular motifs, a conserved transmembrane domain, and a cytoplasmic tail containing a death domain (DD) motif [14]. The DD was originally identified in the TNF-R1 as the region responsible for induction of cell death. The TNF-R1 is expressed on all cells, whereas TNF-R2's expression is limited to lymphocytes. Since the molluscum contagiosum poxvirus infects keratinocytes, our studies will focus on TNF-R1 signaling.

TNF-R1 signaling can result in at least three distinct cellular events: apoptosis, the activation of the cellular NF-κB transcription factor, or the activation of the cellular Jun N-terminal kinase (JNK) transcription pathway [14] (Figure 1.3). It is known that each event correlates with the presence of different adapter proteins forming a "signalsome" at the DD portion of the receptor. For apoptosis, the DD of TNF-R1 binds to TNF Receptor Associated DD protein (TRADD). Next TRADD-Fas-Associated DD (FADD) interactions are followed by FADD-procaspase-8 interactions, auto-activating procaspase-8, effector caspases, and eventually cell death (Figure 1.3). In contrast, the NF-κB activation pathway results in TRADD-RIP (Receptor Interacting Protein) and TRADD-TRAF2 complex formation, which then binds to the DD of TNF-R1. These signaling events lead to activation of IKK, followed by the phosphorylation and degradation of the inhibitor of NF-κB (IκB), allowing NF-κB translocation to the nucleus (Figure 1.3).

Interferon-beta response.

Type 1 interferons, interferon-alpha (IFN-α), and interferon-beta (IFN-β), are critical for anti-viral responses [68]. IFN-β mounts an immediate early response to viral infection, where IFN-α contributes to a delayed response. IFN-β produces the most robust anti-viral response and therefore will be the subject of this review.

dsRNA induced IFN-β expression.

IFN-β expression can be induced by many mechanisms. During poxvirus infection, TLRs and RIG-I are the most robust activators of IFN-β [85]. Specifically,

double-stranded RNA, which is produced during viral gene transcription, is recognized by TLR3 and RIG-I [40, 42, 46, 48]. Though activation of TLR3 and RIG-I occur via different upstream factors, both pathways converge on a kinase complex of TBK-1 and IKKε [29] (Figure 1.4). The TBK-1:IKKε complex is essential for the phosphorylation and activation of IRFs. In turn, IRFs dimerize and migrate to the nucleus and initiate IFN-β expression [30, 34, 50]. This pathway is a common target for viral immune evasion proteins [86]. My work suggests that the MC159 protein may target the TBK-1 complex to inhibit IFN-β (Chapter 3).

IRF proteins are characterized by the presence of two domains, a DNA binding domain (DBD) and a IRF associated domain (IAD) which facilitate either DNA binding or protein:protein interactions respectively [78]. To date, nine IRF proteins have been characterized [78]. However, IFN-β transcription is primarily driven by IRF3 and IRF7 [56, 72]. The IRF3 transcription factor is constitutively expressed in most cell-types, while IRF7 expression must be induced by viral infection or other stimuli [44]. IRF7 activates transcription of both IFN-α and IFN-β, and IRF-3 only activates IFN-β production [78].

In resting cells, IRF3 is sequestered in the cytoplasm because the nuclear localization sequence (NLS) and DBD are in an inactive conformation (Figure 1.4). Upon phosphorylation the IRFs undergo conformational changes that expose the NLS and DBD, allowing IRFs to dimerize. In this confirmation IRFs can translocate to the nucleus and bind the IFN-β promoter. Three families of transcription factors, NF-κB, IRFs, and ATF-2/c-Jun, bind four Positive Regulatory Domains (PRD I-IV) on the IFN-β enhancer [49, 63]. Within the PRDs IRF DBD recognizes the IFN-stimulated Regulatory

Element (IRSE) sequence GAAANN and AANNNGAA. Once bound to IRSE, IRF3 directly interacts with transcriptional cofactors HMG and CBP/p300 to initiate IFN-β expression [54, 58].

IFN-β is secreted once it's produced. Extracellular IFN-β binds to the type I IFN receptors on the virally infected cell and neighboring cells [68](Figure 1.4). Binding of IFN-β to type I IFN receptor results in the activation of Janus activated kinase (JAK)-(STAT) (signal transducer and activator of transcription) signaling cascade resulting in the activation of IRFs and the expression of Interferon Stimulated Genes (ISGs). IFN-β regulates myriad ISGs that together induce an anti-viral state.

The anti-viral state halts viral and cellular processes such as transcription and translation. The three best-characterized ISG are PKR, 2'-5' oligoadenylate synthetases (OAS) and myxovirus resistance gene (Mx) [24, 68]. First, a serine-threonine kinase called PKR, in response to viral dsRNA, phosphorylates the elongation factor eIF2-α, resulting in a block in translation. Secondly, OAS activates ribonuclease L (Rnase L) resulting in the degradation of cellular and viral RNAs. As a secondary anti-viral mechanism, PKR and OAS/RnaseL can slow normal cellular function, i.e. transcription and translation, to a degree that induces apoptosis of virally infected cells. Finally a guanosine triphosphatase (GTPase) called sequesters viral ribonuleoproteins to subcellular compartments.

Due to the potent anti-viral properties of IFN-β many, such as poxviruses herpesviruses and influenzaviruses, have evolved mechanisms to inhibit IRF3 driven expression of IFN-β [72, 76, 86]. I found that MCV encodes multiple proteins capable of inhibiting IFN-β expression (Chapter 3).

4) FLIP regulation of NF-κB, apoptosis and IFN-β

Overview.

Poxviruses have evolved myriad immune evasion proteins of their host's anti-viral responses. These evasion proteins target NF-κB, apoptosis and IFN-β through a variety of molecular mechanisms [24]. Understanding these mechanisms gives immense insight into both viral and host cell biology. In this next section, I will discuss how MC159 and its homologs modulate NF-κB activation and IFN-β expression.

MC159.

The MC159 protein is 241 amino acids in length and is approximately 25 kDa (Figure 1.5). MC159 is characterized the presence of two protein:protein interaction domains called Death Effector Domains (DEDs). These domains were named DED because of their presence in cellular apoptotic proteins e.g. FADD, and procaspase-8. Each DED comprises 6 alpha helices. However in MC159, helix 3 in DED1 is truncated into a rigid loop.

Originally, the MCV MC159 protein was identified for its anti-apoptotic function [5, 73, 83]. Recently, two publications revealed the crystal structure of the MC159 protein [45, 89] (Figure 1.5). Both describe the MC159 protein as having a rigid dumbbell structure in which its two DEDs tightly associate. It is thought that the tandem DEDs facilitate protein:protein interactions.

Anti-apoptosis function.

The MC159 protein is particularly interesting because it has been shown to inhibit either TNF-R1-induced apoptosis or NF-κB activation [59, 73] (Figure 1.6). For apoptosis, the molecular mechanism of the MC159 protein has been intensely studied. Studies published by Garvey *et al.* 2002, show the MC159 protein blocks apoptosis by binding FADD or caspase-8 through an RXDL motif present in each of the MC159 proteins DEDs [25, 26]. Structural studies suggest that the MC159 protein binds FADD, thus preventing its self-oligermization and, in turn, DISC assembly [45, 89]. Additionally, the anti-apoptotic properties of MC159 are bolstered by its interaction with the adaptor protein TRAF3 [80]. Although, there has been debate concerning which binding partners are correlated with apoptosis inhibition, the general mechanism of blocking DISC assembly is accepted.

NF-κB inhibitory function.

Previously, it was shown that the MC159 protein also inhibits TNF-induced NF-κB activation [28]. The molecular mechanism for the MC159 protein NF-κB inhibition is not as well defined as that for apoptosis. Previous studies from our lab revealed that the N-terminal DED of MC159 was sufficient to block NF-κB activation, and that there is a correlation between NF-κB inhibition and TRAF-2 binding [59]. A recent report by Challa *et. al*. suggested that MC159 stimulates NF-κB activation [2]. In our hands MC159 expression can result in low levels of NF-κB activation. However, this activation

is minimal compared to the inhibitory phenotype we consistently observe [59, 66]. Chapter 2 details my work showing that MC159 binds to IKKγ, (not TRAF2), to inhibit NF-κB activation (Figure 1.6).

IFN-β inhibitory function.

More recently, MC159 was shown to inhibit IFN-β expression [3] (Figure 1.6). Since, IFN-β is regulated by multiple transcription factors including IRFs and NF-κB, it was unclear if this inhibition is specific to IFN-β or an indirect result of MC159 mediated inhibition of NF-κB. I found that MC159 could inhibit IFN-β expression independently of NF-κB by blocking the activation of the IRF3 transcription factor (Chapter 3).

MC160.

The MCV MC160 protein is a potent inhibitor of NF-κB activation (Figure 1.6). Despite structural similarity to MC159, MC160 does not to inhibit apoptosis [73]. Similar to MC159, MC160 inhibits NF-κB. The molecular mechanism for MC160 mediated inhibition of NF-κB is well understood. Studies in our lab found that MC160 inhibits NF-κB by two distinct mechanisms [60, 61]. First, MC160 can inhibit procaspase-8 induced NF-κB activation by binding to procaspase-8. MC160 also interacts with chaperon HSP90 to destabilize IKKα and block NF-κB activation. Although, MC160-HSP90 interactions are well understood for MC160 mediated NF-κB inhibition, little is known concerning how these interactions affect other pathways, such as interferons. Here I show that MC160 inhibits IFN-β expression (Chapter 4).

ORFK13.

Viral FLIPs are not limited to poxviruses. Human Herpes Virus 8, which is the etiological agent of Karposi's sarcoma, also encodes a vFLIP called K13. The K13 protein shares structural and sequence homology with the MC159 protein (Figure 1.6). Similarly to MC159, K13 can inhibit apoptosis by blocking caspase-8 activation [20, 39]. However, unlike MC159, K13 activates NF-κB during infection to promote cell survival, transformation, proliferation, and cytokine secretion [13, 77]. The expression of K13 by HHV8 during latency plays a critical role in viral oncogenesis. For this reason, the molecular mechanism of K13 has been the subject of intense investigation. Initial studies suggested that K13 targeted the adaptor proteins TRAF2 and TRAF3 in order to activate NF-κB [13, 29]. Further studies revealed that K13 targets the IKK complex via its n-terminal DED, which directly interacts with the IKKγ subunit [22, 51]. These interactions result in activation of the catalytic subunits IKKα and IKKβ. To date, it is unclear whether K13's function extends to pathways outside of NF-κB and apoptosis.

Recently a K13:IKKγ co-crystal structure was published. This study shows K13 binding to IKKγ via 2 open clefts within K13's DED1 [2]. The authors predicted that these clefts are closed in the MC159 protein's structure therefore preventing MC159 from binding to IKKγ. However we have experimental evidence illustrating that MC159 can associate with IKKγ (Chapter 2).

5) Thesis outline

The MC159 protein is a potent inhibitor of apoptosis and NF-κB activation. The goal of my thesis project was to characterize the molecular mechanism of MC159 mediated NF-κB inhibition. Based on previous studies in our lab, we hypothesized that MC159 interacted with TRAF2 to block NF-κB activation.

My findings suggest that TRAF2 and TRAF3 interactions are dispensable for MC159 mediated inhibition of NF-κB (Chapter 2). I also found that MC159 targets the IKK complex through interactions with the IKKγ subunit. I showed that MC159 interactions occur in the context of viral infection using either a surrogate virus or MCV preparations, and I demonstrated that RXDL motif within DED1 of the MC159 protein is necessary for inhibition.

Chapter 3 outlines the characterization of a novel function for the MC159 and vFLIP proteins in the inhibition of IFN-β expression. Here I demonstrate that MC159, MC160, and cFLIP (but not K13) can block IFN-β expression. Interestingly, I found that IFN-β expression was inhibited in an NF-κB independent manner. I found that MC159 could prevent phosphorylation of the TBK-1 kinase, suggesting that MC159 may act on the TBK-1: IKKε complex. Unlike MC159's inhibition of NF-κB, MC159 mutant proteins with a single DED (either DED1 or DED2) and the C-terminal tail were able to inhibit IFN-β expression. This suggests that either DED is sufficient for inhibition or the c-terminal tail is mediating inhibition. I hypothesized that MC159 was using TRAF3, an adapter protein involved in IFN responses, binding sites in its c-terminus to inhibit IFN-β. However, MC159 mutants that were deficient in TRAF3 binding retained function.

Chapter 4 details the mechanism in which MC160 inhibits IFN-β expression. Using various deletion mutants I demonstrate the MC160 can inhibit IFN-β using either its DED or C-terminal tail. Due to the multiple regions of MC160 capable of inhibition, I hypothesize that MC160 uses two mechanisms to inhibit IFN-β expression. Further study is needed to characterize the molecular mechanisms of this inhibition.

The initial goal of my thesis was to characterize the molecular mechanism for MC159 mediated inhibition of NF-κB. However, the scope of my project grew beyond the NF-κB pathway to include IFN-β. The MC159 protein has extremely potent anti-inflammatory properties blocking three arms of the anti-viral response, apoptosis, NF-κB, and IFN-β. Uncovering the molecular mechanisms behind these functions gives great insight into MCV and host biology.

6) Figures

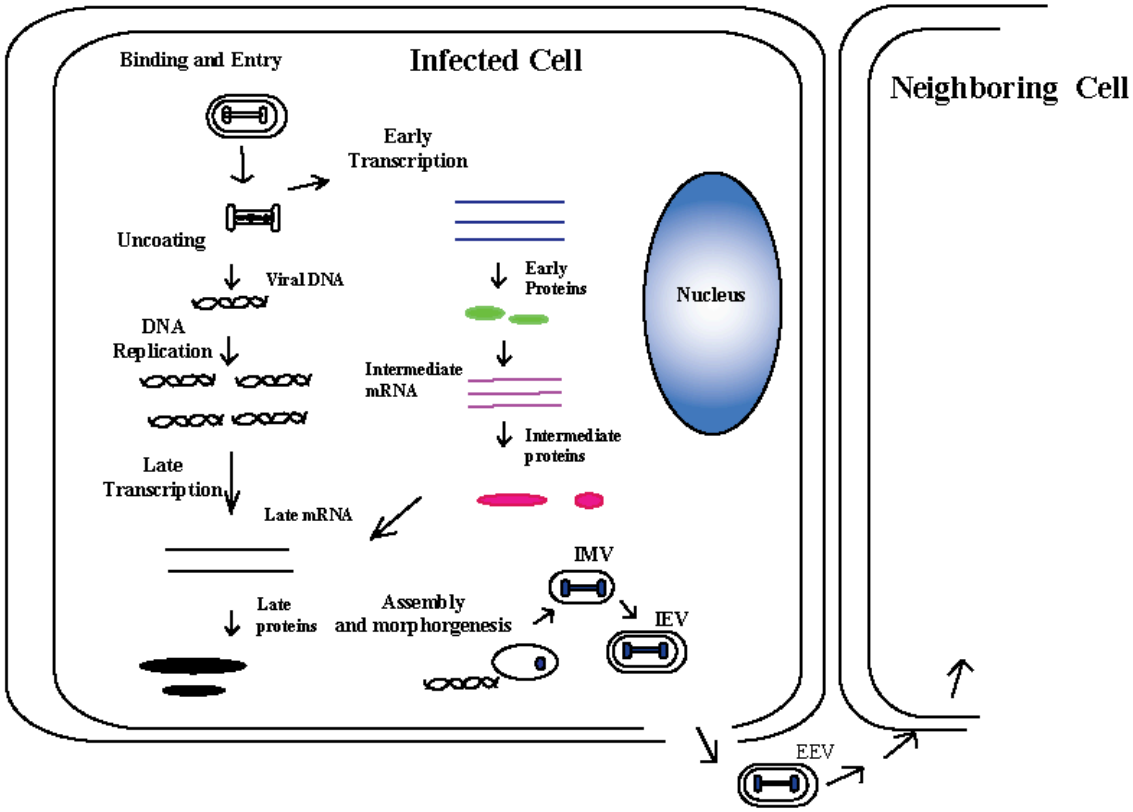

Figure 1.1 Poxvirus replication cycle. Upon entry into the host cell the initiation of transcription and translation of early genes occurs. Next uncoating and replication of the dsDNA genome occurs followed by the transcription and expression of intermediate and late genes. New viral particles are then assembled and morphogenesis results in the release of new EEVs (extracellular enveloped viruses) particles leave the host cell. These EEVs can go on to infect neighboring cells. (See text for more detail)

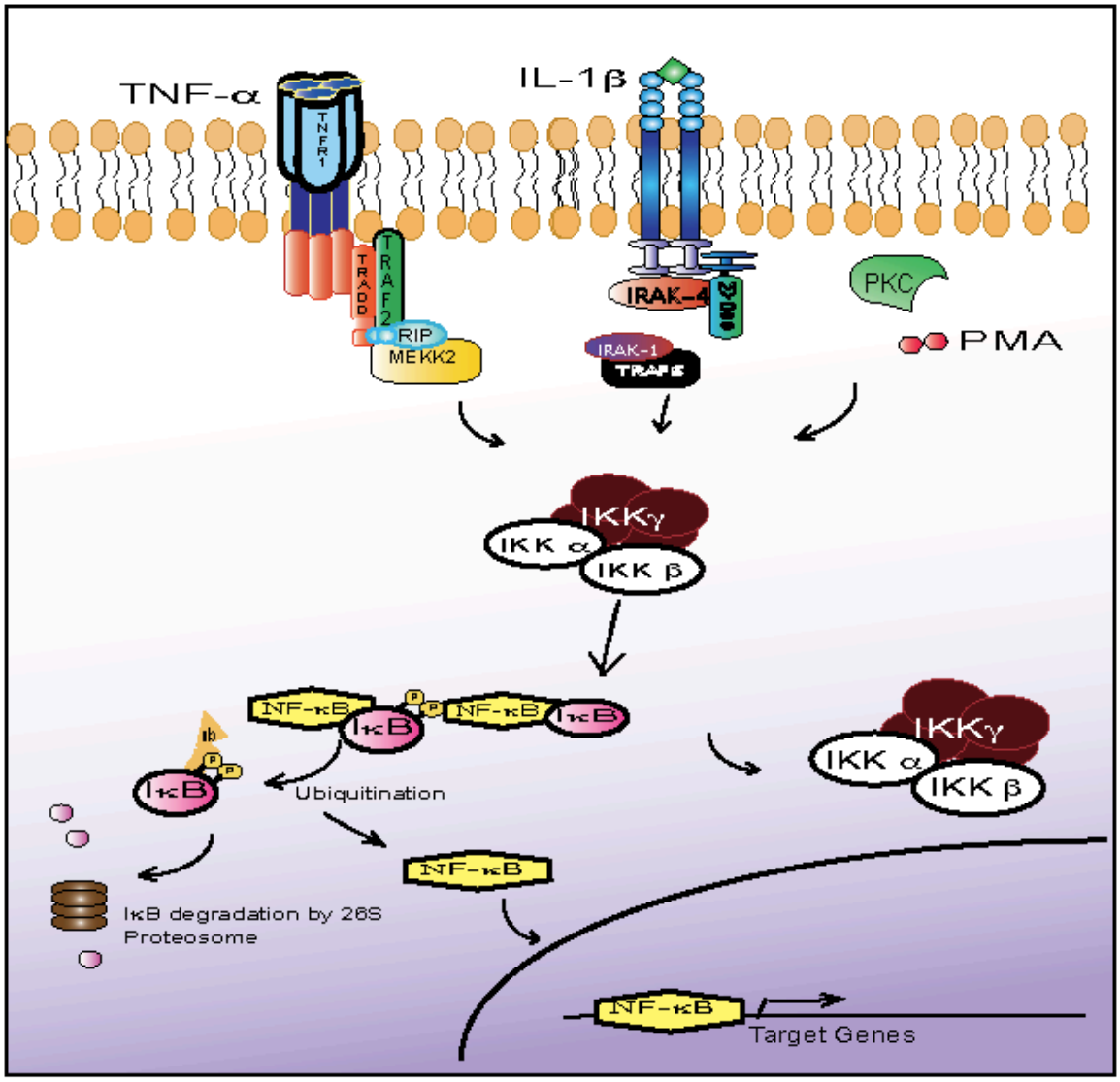

Figure 1.2 Canonical pathways to NF-κB activation. Activation of NF-κB induced by cytokines, stress and PAMPs (Pathogen Associated Molecular Patterns) results in the activation of the IKK complex. The IKK complex goes on to phosphorylate the inhibitor of NF-κB (IκB) targeting it for degradation by the proteosome. Then NF-κB translocates into the nucleus and activates transcription of target genes.

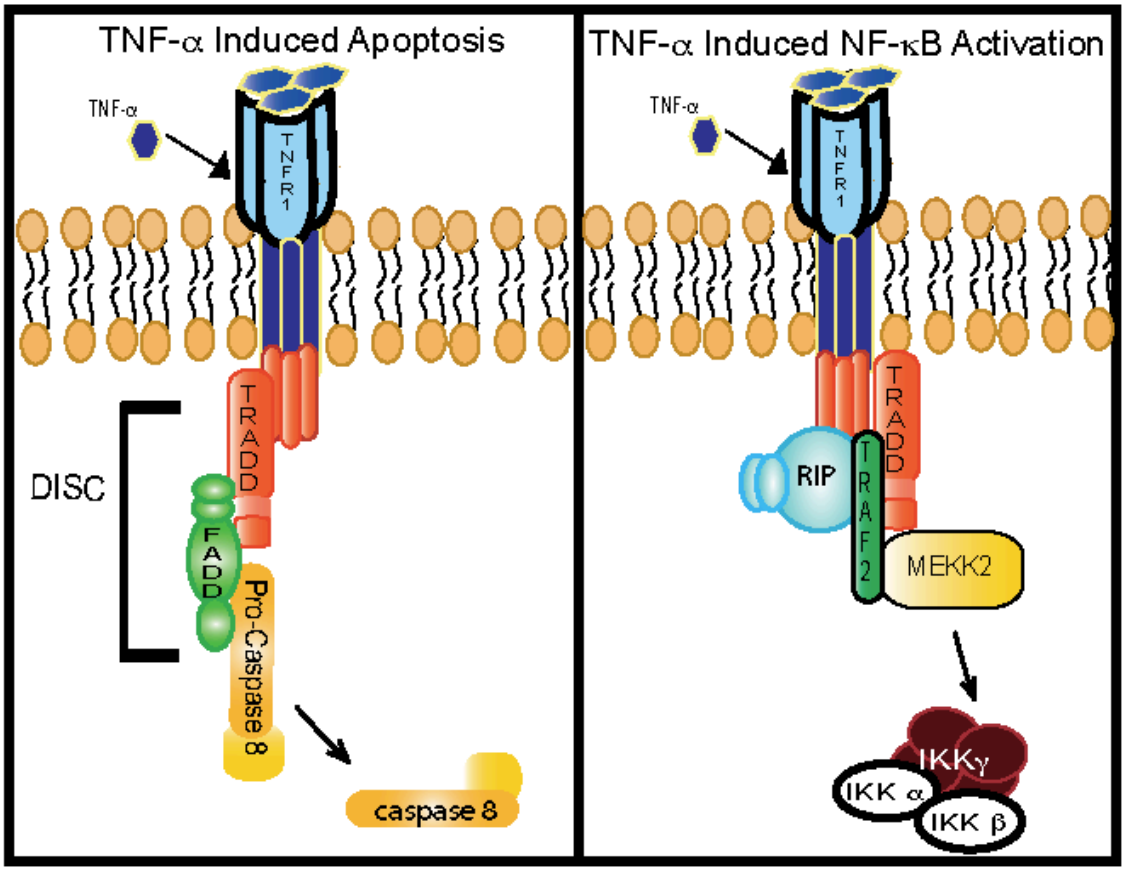

Figure 1.3 Summary of the apoptosis and NF-κB signaling pathways downstream of TNF-R1. For apoptosis, TNF binds TNF-R1 and TRADD, FADD and procaspase-8 are recruited to the receptor, forming a Death Inducing Signaling Complex (DISC), which results in caspase-8 activation. For NF-κB activation TNF binds TNF-R1 resulting in the recruitment of TRADD, TRAF2, RIP and MEKK2 to the receptor. This complex recruits and activates the IKK complex.

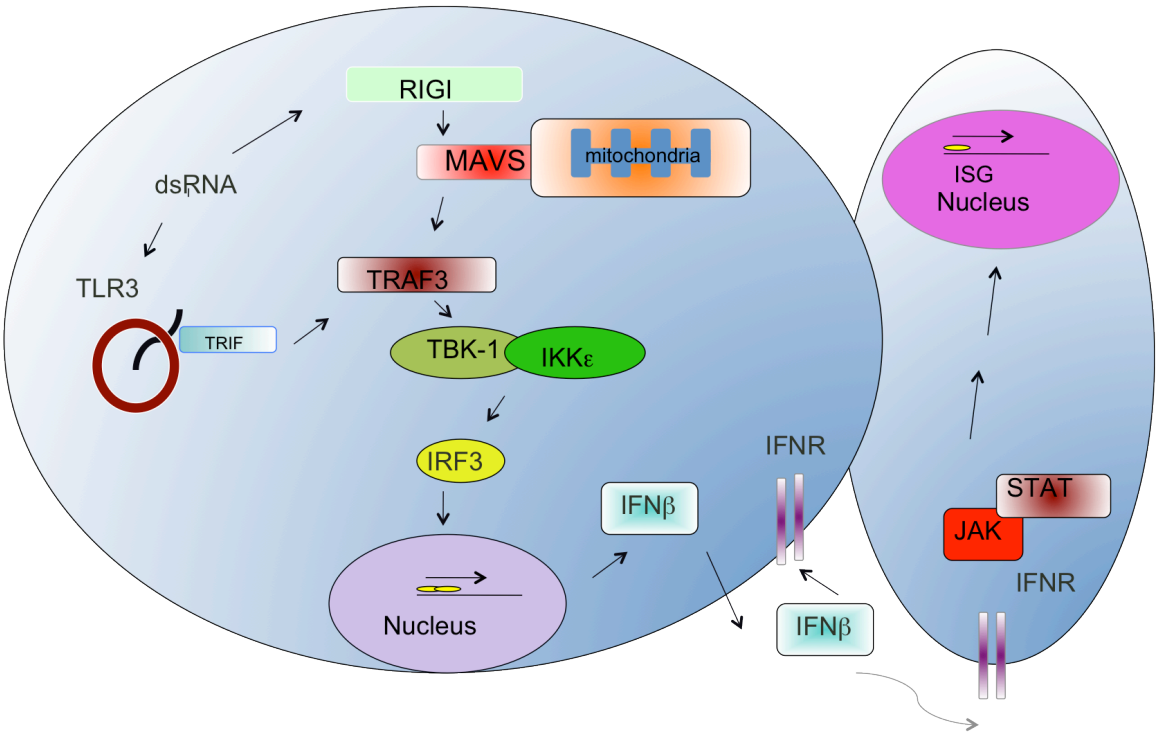

Figure 1.4 Activation of IFN-β expression and Interferon Stimulated Genes (ISG) expression. Viral replication results in the production of dsRNAs that are recognized by TLR3 and RIG-I. Both TLR3 and RIG-I use TRAF3 to recruit the TBK-1:IKKε complex. The TBK-1:IKKε complex phosphorylates the IRF3 transcription factor allowing it to dimerize, enter the nucleus and drive expression of IFN-β. Then IFN-β is secreted and binds to IFN receptor (IFNR). This activates the JAK/STAT pathway leading to the expression of ISGs.

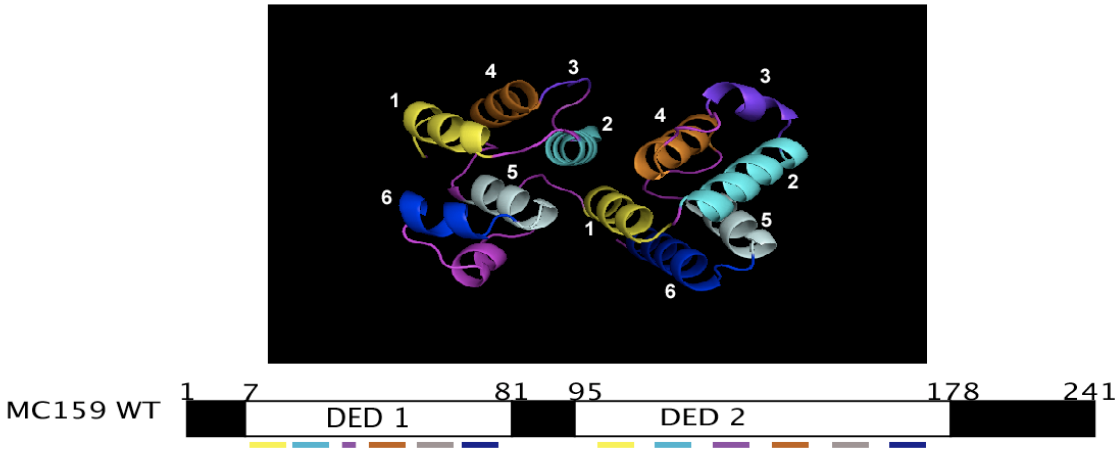

Figure 1.5 Crystal structure and linear diagram of the MC159 protein. The MC159 protein contains two Death Effector Domains. Each DED is made up of six alpha helices labeled one through six (yellow, cyan, purple, orange, gray and blue). The crystal structure contains both DEDs connected by the linker region. The linear MC159 diagram shows the full-length protein including the N- and C-termini of the protein.

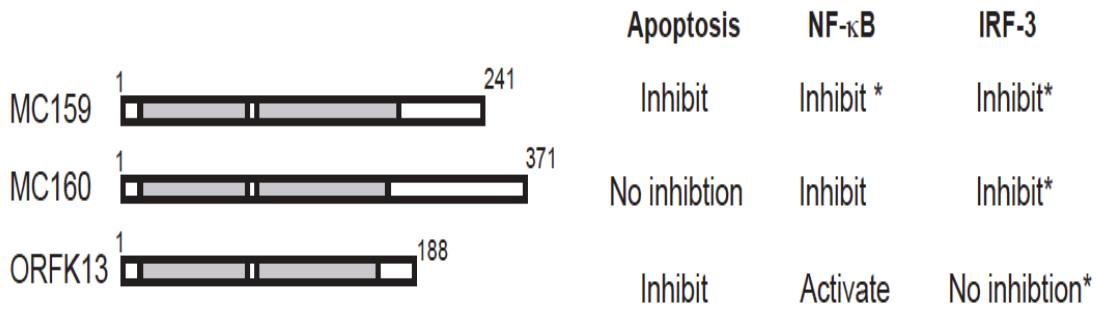

Figure 1.6 Diagram and summary of vFLIPs and their modulation of apoptosis, NF-κB and IRF-3. The MCV MC159 protein is able to inhibit apoptosis, NF-κB and IRF-3 activation. The MCV MC160 protein is able to inhibit NF-κB and IRF-3.

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Chapter 2: The MC159 protein from the molluscum contagiosum poxvirus inhibits NF-κB activation by interacting with the IκB kinase complex

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1) Introduction

The molluscum contagiosum virus (MCV) belongs to the *Poxviridae* family [2, 38, 39]. Other than the variola virus (the causative agent of smallpox), MCV is the only poxvirus that strictly infects humans. MCV infection is limited to keratinocytes, causing a persistent dermatological infection (molluscum contagiosum; MC) that results in the formation of benign self-limiting neoplasms [44]. MC occurs worldwide [26, 27, 36], and reports show 8-23% of humans test positive for anti-MCV antibodies [24, 45]. Lesions can persist for 6-9 months in healthy individuals and can last for many years in immunocompromised individuals [44].

MCV-containing lesions are larger in immunocompromised people [1], suggesting that the immune response does indeed detect and attempt to restrict viral replication and pathogenesis. Indeed, multiple cytokines and immune cells are present at MCV lesions. Of note, TNF is highly expressed at MCV lesion sites [25]. It is thought that TNF is important to control MCV infections because patients receiving anti-TNF therapy have increased incidents of MCV infections [9].

TNF is a potent activator of NF-κB, a cellular transcription factor that regulates immune responses during infection [8, 16, 17]. TNF binding to TNF-R1 initiates the

formation of a signalsome containing the adaptor proteins TRADD, TRAF2 and RIP [8]. The formed signalsome then recruits and activates the IKK complex, a convergence point for canonical activators of NF-κB [31]. The IKK complex is composed of three major subunits; two catalytically active kinases (IKKα and IKKβ) and a regulatory subunit (IKKγ). Inactive NF-κB resides in the cytoplasm bound by its inhibitor, IκB. Upon stimulation of the NF-κB pathway, IKK becomes activated, and phosphorylates an IκB protein [16, 17]. Phosphorylation of IκB results in its ubiquitination and subsequent degradation. Next, freed NF-κB translocates to the nucleus, where it binds to a unique sequence and activates the transcription of its target genes.

Like all poxviruses, MCV encodes myriad immunoevasion molecules, and these molecules are thought to act as virulence factors to neutralize localized immune responses [38, 39]. Unfortunately, a cell culture or animal model for MCV is not available, making it difficult to directly test the effect of an MCV protein on virulence. Nevertheless, much information about the function of MCV proteins has been learned by expressing them independent of MCV [33]. MC159 is a well-studied MCV immunoevasion protein. MC159, and the closely related MC160 protein, are viral FLIPs [33]. Like other cellular and viral FLIPs, MC159 contains two tandem protein-protein interaction domains called death effector domains (DEDA, DEDB) [28, 47]. MC159 inhibits TNF-induced NF-κB activation [34]. To further investigate the MC159 protein's molecular mechanism, we evaluated the effect of MC159 on other well-characterized NF-κB activation pathways. Interestingly, the MC159 protein also inhibited PMA-induced NF-κB activation or NF-κB activation due to MyD88 over-expression, suggesting MC159 inhibits a signaling event downstream of the TNF-R1 signalsome. Using

immunoprecipitations in cells lacking IKK subunits, we demonstrate that the MC159 protein co-immunoprecipitates with the IKKγ subunit of the IKK complex. Previous studies in our lab revealed that the N-terminal DED (DEDA) of MC159 was sufficient to block NF-κB activation, and also mediates MC159-TRAF2 interactions [34]. Here, using MC159 proteins containing substitution mutations within DEDA, data failed to reveal a correlation between MC159-TRAF2 interactions and MC159's inhibitory function, suggesting that only MC159-IKK interactions are relevant for this inhibitory function of MC159.

2) Materials and methods

Cell culture, plasmids, viruses.

Human embryonic kidney cells (HEK293T; 293T) and human lung fibroblast cells (MRC-5) were obtained from the American Type Culture Collection. Wild-type, IKK α –/– and IKK β –/– mouse embryonic fibroblast cells (MEF) were obtained from Dr. Michael May at the University of Pennsylvania [41]. IKKγ -/- MEFs were obtained from Dr. Tak Mak. All cells were cultured in Eagles's minimum essential medium supplemented with 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Plasmid pMC159 consists of the MC159 gene inserted into vector pCI [40]. Plasmid pMC159 A encodes a MC159 gene that lacks the C-terminal DED (DEDB) [13]. Plasmid pMC159 B encodes a MC159 gene that lacks the N-terminal DED (DEDA) [13]. Plasmid pMC159 21 containing a MC159 gene with substitution mutations where

residues L72, L73 and were altered to A [14]. Plasmid pMC159 24 contained a MC159 gene where residue L31 was altered to an A [14]. Plasmid pHA-TRAF2 encodes an (HA) epitope-tagged TRAF2 protein, and was provided by Dr. Jonathan Ashwell, (National Institute of Health) [29]. Plasmid pFLAG-MyD88, which produces a FLAG epitope-tagged MyD88 protein, was a kind gift from Dr. Richard Tapping (University of Illinois). Dr. Margot Thome, (University of Lausanne), provided plasmids pMC159 DM and pMC159 Δ, which encode MC159 proteins with mutated (DM) or deleted (Δ) TRAF3 binding sites, respectively [43]. For all experiments involving plasmids, DNA was transfected into cells using FuGene 6 transfection reagent (Roche; following manufacturers protocol).

Lesions containing MCV used for immunoprecipitation experiments were collected from human donors. Each MCV lesion was dounce homogenized, suspended in 1 ml of 1mM Tris and stored at -70^{o}C [2].

A recombinant vaccinia virus (VACV) expressing the MC159 protein (vMC159) is as described [40]. Briefly, MC159 cDNA was stably introduced into a VACV lacking the B13R gene (vcrmA-). Thus as a control, some cells were infected with vcrmA- virus. All viral infections of cells were performed in Eagle's minimal essential medium supplemented with 2 mM L-glutamine, 2.5% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Luciferase assays.

Luciferase reporter assays were performed as previously described [34]. For all luciferase assays, subconfluent 293T cellular monolayers were co-transfected with two

reporter plasmids, pNF-κB (225 ng; Stratagene) and pRL-null (25 ng; Promega). For luciferase assays in which cells were treated with TNF (10 ng/ml; Roche), subconfluent 293T cellular monolayers were co-transfected with pNF-κB, pRL-null and 750 ng either pCI, pMC159, pMC159 A, pMC159 B, pMC159 21, pMC159 24, pMC159 DM or pMC159 Δ. At 24 h post-transfection, cells were incubated with medium either containing or lacking TNF (10 ng/ml) for 8 or 12 h. Next, cells were lysed in 1 x passive lysis buffer (PLB) (Promega) and analyzed for firefly and sea pansy luciferase activities. In other luciferase reporter assays in which PMA (Promega) was used, subconfluent 293T cellular monolayers were co-transfected with pNF-κB, pRL-null and 750 ng of either pCI, pMC159, pMC159 A or pMC159 B. At 24 h later, cells were incubated with medium containing or lacking PMA (50 ng/ml) for 8 h. Next, cells were lysed in 1 x PLB and analyzed for luciferase activities. In another set of experiments monolayers of subconfluent 293T cells were co-transfected with pNF-κB, pRL-null, 500 ng of pMyD88 and 750 ng of either pCI, pMC159, pMC159 A, pMC159 B. At 24 h post-transfection, cells were lysed in 1 x PLB and analyzed for luciferase activities.

All luciferase activities were measured as relative light units using a BioTek luminometer and the Dual Luciferase Reporter Assay System (Promega), with the exception of luciferase assays shown in Figure 1, in which the Luminoskan Microplate Luminometer (Labsystems) was used. All experiments were performed in triplicate. Relative luciferase activity ratios were calculated by dividing firefly luciferase activities by sea pansy luciferase activities. Luciferase activity ratios were then normalized to that of pCI-transfected cells incubated in regular medium, and this value was set as 1.

Statistical significance was calculated using a Student's *t*-test where $p < 0.05$. Cellular lysates from luciferase assays were analyzed for protein expression by immunoblotting (see below).

Immunoprecipitations.

To detect MC159-TRAF2 interactions, 1 well of a 6-well tissue culture plate of 293T cells was co-transfected with 1000 ng pHA-TRAF2 and 1000 ng pCI, pMC159, pMC159 B, pMC159 21 or pMC159 24. At 24 h post-transfection, cells from each well were removed from the plate by scraping, and collected by centrifugation (1,000 x g for 10 min). Cellular pellets were resuspended in 500 μl DED lysis buffer for 30 min at 4°C [14]. Cellular lysates were centrifuged (14,000 x g for 10 min). Clarified supernatants were collected. 50 μl of the supernatants were saved, and 30 μg of protein from this sample (approximately 15 μl) was used to assess expression levels of proteins. The remaining 450 μl of clarified lysates were incubated overnight with 1 μg of monoclonal mouse anti-HA (Sigma Aldrich) and 50 μl protein G-Sepharose (Invitrogen) beads with constant rotation at 4°C to detect MC159-TRAF2 interactions. To detect MC159-IKKγ interactions, 1 well of a 6-well plate of 293T cells was transfected with 1000 ng of either pCI or pHA-MC159. At 24 h post-transfection, cells from each well were removed from the plate by scraping, and collected by centrifugation (1,000 x g for 10 min). Cellular pellets were resuspended in 500 μl DED lysis buffer for 30 min at 4°C [14]. 50 μl of the supernatants were set aside, and 30 μg of protein from this sample (approximately 15 μl) was used to assess expression levels of MC159 and IKKγ. The remaining 450 μl of clarified lysates were incubated overnight with 1 μg of monoclonal mouse anti-IKKγ

(sc-56919; Santa Cruz) antibodies to detect MC159-IKKγ interactions and 50 μl protein G-Sepharose (Invitrogen) beads with constant rotation at 4°C. As a control, an identical set of clarified lysates were incubated instead with 1 μg of mouse IgG (Sigma Aldrich) antibodies and 50 μl protein G-Sepharose (Invitrogen) beads with constant rotation at 4°C

In other immunoprecipitation experiments in which mouse embryo fibroblasts (MEFs) were used, 10-cm^2 dishes of subconfluent monolayers of WT mouse embryo fibroblasts (MEFs) or IKKα -/-, IKKβ -/- or IKKγ -/- MEFs were transfected with 1000 ng of pCI, pMC159 or pMC159 B. At 24 h post-transfection, cells were dislodged from plates by scraping into 1 ml chilled PBS, and collected by centrifugation (1,000 x g for 10 min). Cellular pellets were incubated in 150 μl DED lysis buffer for 30 min at 4°C [14]. Cellular lysates were centrifuged (14,000 x g for 10 min). 50 μl of the supernatants were set aside, and 30 μg of protein from this sample (approximately 5 μl) was used to assess expression levels of MC159 and IKK proteins. The remaining 100 μl of clarified lysates were incubated overnight with 2 μg of polyclonal rabbit anti-IKKγ (sc-8330, Santa Cruz) antibody or 3 μg of anti-IKKβ antibody (Imgenex) for 1 h with constant rotation at 4°C. Following the incubation with anti-IKKγ, 50 μl protein G-Sepharose beads were added to each of the immunoprecipitation reactions and incubated at 4°C for 1 h with constant rotation.

Immunoprecipitations also were performed using lysates from virus-infected cells. For these experiments, 1 well of MRC-5 cells in a 6-well plate were either mock-infected or infected with 300 μl of an MCV lesion preparation, a volume of MCV preparation that produced cytopathic effects in MRC-5 cells within 24 h. Alternatively, when using 293T cells, 1 well of cells from a 6-well plate were mock-infected or infected

with vcrmA- or vMC159 (MOI=10) [40]. For both experiments, at 24 h post-infection, cells were dislodged from plates by scraping and collected by centrifugation (1,000 x g for 10 min). Cellular pellets were incubated in 500 μl DED lysis buffer for 30 min at 4°C [14]. Cellular lysates were centrifuged (14,000 x g for 10 min). 50 μl of the supernatants were set aside, and 30 μg of protein from this sample (approximately 15 μl) was used to assess expression levels of MC159 and IKK proteins. The remaining 450 μl of clarified lysates were incubated with either 2 μg of a monoclonal mouse anti-IKKγ (sc-56919; Santa Cruz) antibody or mouse IgG (Sigma Aldrich) for 1 h with constant rotation at 4°C. Following incubation with anti-IKKγ, 50 μl protein G-Sepharose beads were added and supernatants were incubated for 1 h with constant rotation at 4°C.

For all immunoprecipitations, supernatant bead mixtures were pelleted by centrifugation (14,000 x g / 30 s) and washed 3 times with DED lysis buffer. Pelleted bead-protein complexes were suspended in 30 μl of 2x Laemmli buffer containing 5% 2-mercaptoethanol, and boiled for 5 min.

Immunoblotting and antibodies.

The protein concentration of each clarified cellular lysate was determined using bicinchoninic acid assay (BCA; Pierce). Approximately 30 μg of protein from each lysate sample was prepared for immunoblotting by resuspending in non-reducing lane marker sample buffer (Thermo Scientific) with 5% 2-mercaptoethanol and boiling for 5 min.

Immunoprecipitated samples or clarified cellular lysates were subjected to 12% SDS-PAGE and proteins were subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were incubated with indicated primary

antibodies (see below), washed three times in TBS-T, and incubated with appropriate HRP-conjugated secondary antibodies. Immunoblots were developed using Pierce super signal west chemiluminescence reagent. Primary antibodies used in these experiments were: polyclonal rabbit anti-IKKγ (1:500, sc-8330, Santa Cruz), polyclonal rabbit anti-MC159 (1:1000) [40], monoclonal mouse anti-HA (1:500, Sigma Aldrich), monoclonal mouse anti-IKKγ (1:500, sc-56919, Santa Cruz), monoclonal mouse anti-IKKα (1:500, Imgenex), or monoclonal mouse anti-IKKβ (1:500, Imgenex). Monoclonal mouse anti-E3 (1:500) antibody was a kind gift from Dr. Stuart Isaacs (University of Pennsylvania) [46]. Secondary antibodies conjugated to horseradish peroxidase were obtained from either Thermo Scientific (goat anti-mouse IgG) or Calbiochem (goat anti-rabbit IgG).

3) Results

The MC159 RXDL motif confers resistance to TNF-induced NF-κB activation.

The N-terminal DEDA of MC159 is necessary for inhibition of TNF-induced NF-κB [34]. To further elucidate the regions of the DED involved in NF-κB inhibition, we assayed the function of two MC159 proteins with substitution mutations in discrete motifs within DEDA; the RXDL motif (pMC159 21) and a hydrophobic patch (pMC159 24) [13, 14]. Each mutant protein was assessed for its ability to inhibit TNF-induced NF-κB using a luciferase reporter assay (Fig. 1). Similar to a previous report [34], the wild-type MC159 protein inhibited TNF-mediated NF-κB activity, as seen by a 4-fold decrease in luciferase activity compared to cells transfected with empty vector (pCI). The RXDL motif was important in providing inhibitory function because TNF treatment

of cells resulted in luciferase activity levels in pMC159 21-transfected cells that were similar to that of pCI-transfected cells. In contrast, pMC159 24-transfected cells exhibited a 10-fold decrease of luciferase activity compared to cells transfected with pCI. Differences in luciferase activity levels were independent of MC159 protein expression levels (Figure 2.1, bottom panel). These data suggest that the RXDL motif, but not the hydrophobic patch in DED A-mediated inhibition of NF-κB activation.

The regions of MC159 responsible for interactions with either TRAF2 or TRAF3 are dispensable for NF-κB inhibitory function.

Since the MC159 DEDA is sufficient to inhibit NF-κB activation, and MC159 A also immunoprecipitates with TRAF2, a TNF-R1 signaling adaptor protein, this suggests that MC159-TRAF2 interactions are required for the MC159 inhibitory function [34]. To test this hypothesis, the mutant MC159 21 and MC159 24 proteins were assayed for their ability to co-associate with TRAF2 using immunoprecipitations (Figure 2.2). Similar to the wild-type MC159 protein, the MC159 21 and MC159 24 proteins co-associated with HA-TRAF2 (Figure 2.2, top panel) indicating that MC159-TRAF2 interactions did not correlate with inhibition of NF-κB. The MC159 B protein, which lacks DEDA, no longer immunoprecipitated with TRAF2, as previously reported. Similar levels of expression of the TRAF2 and MC159 proteins were detected by immunoblotting (Figure 2.2, middle panel and bottom panel, respectively). It should also be noted that the MC159 protein was not detected from samples in which the HA-TRAF2 protein was absent, suggesting that the MC159 protein does not non-specifically bind to protein G sepharose beads.

An earlier report by Thurau et al. established that TRAF3-MC159 interactions stabilized TRAF2-MC159 interactions [43]. If TRAF3 stabilizes MC159-TRAF2 interactions to aid in the inhibition of NF-κB, then we hypothesized that MC159 proteins that do not bind to TRAF3 would not inhibit NF-κB. Accordingly, two mutant MC159 proteins (MC159 DM and MC159 Δ) that are deficient in TRAF3 binding [43] were assessed by a luciferase reporter assay for their ability to inhibit TNF-induced NF-κB activation (Figure 2.3, graph). The inhibition of NF-κB activation by either MC159 or MC159 DM was statistically significant ($p < 0.05$), with wild-type MC159 inhibiting luciferase activity 3.4-fold, and MC159 DM inhibiting 3.4-fold. While luciferase activity levels in MC159 Δ–expressing cells were lower than in pCI-transfected cells, the difference in levels was not statistically significant. The similar expression levels of each MC159 protein were confirmed by immunoblotting of the cellular lysates (Figure 2.3, bottom panel). Unfortunately, the MC159 Δ protein lacks the epitope recognized by the anti-MC159 antibody [40], and therefore was not detectable by this method. Our results indicate that TRAF2 and TRAF3 interactions are insufficient to mediate inhibition of NF-κB by the MC159 protein.

MC159 DEDA inhibits TNF-, PMA- or MyD88-induced NF-κB activation.

While many external stimuli trigger different upstream NF-κB activation pathways, most NF-κB pathways converge on IKK activation. We observed that the wild-type MC159 protein inhibited luciferase activity in response to three stimuli (TNF, PMA and MyD88 over-expression) that use different upstream signal transduction pathways to stimulate IKK and NF-κB (Figure 2.4). In this case, the MC159 inhibitory

function was about a 2.8-fold decrease from pCI-transfected cells, in comparison to Figure 2.1, in which MC159 inhibited 4-fold as compared to pCI-transfected cells. MC159 A also decreased luciferase activity to in response to TNF (1.8-fold), PMA (2.3-fold) or MyD88 (2.3-fold) (Fig. 4A-C), albeit not to the extent of wild-type MC159. However, the MC159 B protein did not significantly decrease luciferase activity under any condition tested. A similar level of MC159 protein expression was observed for all three experiments as shown by immunoblotting (Figure 2.4A-C). Thus, the MC159 protein utilized DEDA to inhibit NF-κB activation by inhibiting an event that is conserved amongst all three signaling pathways.

The MC159 protein co-immunoprecipitates with IKKγ, and this association occurs regardless of the presence or absence of IKKα or IKKβ.

Most inducers of canonical NF-κB signaling require IKK activation [31]. Since MC159 inhibited several NF-κB inducers, we hypothesized that the MC159 protein interacts with one or more of the components of the IKK complex. MC159 was reported to not co-immunoprecipitate with either IKKα or IKKβ [6, 7]. Moreover the Kaposi's sarcoma herpes virus (KSHV) K13 protein, an MC159 homolog, was shown to bind to IKKγ [12]. As a result of these data, we queried if MC159 interacted with IKKγ, evaluating whether MC159 and IKKγ could be co-immunoprecipitated in MC159-expressing cells. As shown in Figure 2.5A, the IKKγ protein co-associated with MC159 in pHA-MC159-transfected cells, but was not immunoprecipitated from cells transfected with empty vector (pCI) (Figure 2.5A). Similarly, immunoprecipitations using an anti-IKKγ antibody resulted in MC159-IKKγ co-associations from pMC159-transfected cells,

but not pCI-transfected cells (Figure 2.5B). If an antibody that did not detect MC159 or IKKγ was used for immunoprecipitations, the MC159 protein was no longer detected in immunoprecipitated samples (Figuire 2.5C), indicating that the MC159-IKKγ co-immunoprecipitations were specific.

The above experiments were performed using the 293T human cell line. We next assessed whether these same interactions occurred in mouse embryo fibroblast cell (MEFs) that were transiently transfected with plasmids lacking or containing the MC159 gene. As shown in Figure 2.6, MC159 still co-immunoprecipitated with IKKγ. Notably, the MC159 B protein, which did inhibit NF-κB, did not co-immunoprecipitate with IKKγ, suggesting MC159 interaction with the IKK complex is critical for MC159's inhibitory function.

Regardless of the presence or absence of MC159, both the IKKα and IKKβ proteins co-immunoprecipitated with IKKγ in wild-type MEFs (Figure 2.6). It should be noted that, because the transfection efficiency of MEFs is less than 100%, it is impossible to assess whether co-immunoprecipitated IKKα and IKKβ proteins were from cells lacking or containing MC159 molecules, making it difficult to conclude whether MC159 affects IKK complex formation.

To evaluate the importance of the presence of IKKα, IKKβ or IKKγ for co-immunoprecipitating MC159, coimmunoprecipitations were performed with pMC159-transfected MEFs from either IKKα (IKKα -/-), IKKβ (IKKβ -/-) or IKKγ (IKKγ -/-) knockout mice. MC159 still co-immunoprecipitated with IKKγ when probing lysates from either IKKα -/- or IKKβ -/- cells (Figure 2.7A). These data suggest that either IKKα or IKKβ are not directly involved in MC159 binding, or that the absence of either IKKα

or IKKβ can be compensated for. The presence of MC159 did not prevent IKKγ-IKKα or IKKγ-IKKβ co-immunoprecipitations, suggesting that MC159 did not grossly alter the IKK complex. Comparable levels of MC159, IKKα, IKKβ and IKKγ protein expression in cellular lysates were shown by immunoblotting (Figure 2.7A), indicating that MC159 protein expression did not affect the expression of these proteins.

Figure 2.7B utilized IKKγ -/- MEFs to assess the importance of IKKγ in MC159-IKK complex association. While MC159 co-immunoprecipitated with IKKβ in wild-type MEFs, this interaction was no longer detected in IKKγ -/- cells. Thus, the IKKγ molecule is necessary for MC159 to interact with the IKK complex.

The MC159 protein co-immunoprecipitates with IKK during poxvirus infection.

To evaluate MC159-IKK interactions in the context of a poxvirus infection, immunoprecipitations were performed using lysates from cells infected with either a recombinant vaccinia virus expressing (vMC159) or the parental vaccinia virus (vcrmA-). Similar to results from assays in which the MC159 protein was expressed by transfection, MC159 co-immunoprecipitated with IKKγ in cells infected with vMC159, but not in cells infected with a virus lacking the MC159 gene (vcrmA-) or mock-infected cells (Figure 2.8A, top panel). The expression of E3, an early vaccinia virus protein, demonstrated vcrmA- and vMC159 early protein expression in 293T cells (Figure 2.8A, bottom panel). Identical sets of lysates from virus-infected cells were incubated with IgG antibodies that were not specific for IKKγ. MC159 proteins were no longer detected in the immunoprecipitates from these reactions (Figure 2.8B), indicating that MC159 did not promiscuously bind to protein G sepharose beads.

It is known that MCV infection of MRC-5 cells results in abortive infection, but the early and late classes of poxviral proteins are still expressed [2, 3]. To evaluate MC159-IKKγ interactions during MCV infection, MRC-5 cells were infected with a MCV lesion preparation, and cell lysates were assayed for MC159-IKKγ interactions by using co-immunoprecipitation. MC159 co-immunoprecipitated with IKKγ, albeit in low levels, in cells infected with MCV preparations but not in mock-infected cells (Figure 2.8B, top panel). Additionally, MC159 and IKKγ proteins were detected in cellular lysates (Fig. 8B, bottom panel). Thus, a conclusion from this data is that the MC159 protein coimunoprecipitates with IKK (Figure 2.9).

4) Discussion

The IKK complex plays a crucial role in NF-κB activation, making it an appealing target for viruses as a means to block NF-κB-mediated activation of the pro-inflammatory response [18, 23, 32]. Other viral proteins target the IKK complex to inhibit NF-κB activation, such as the vaccinia virus B14 protein and the enterovirus 2C protein, each of which bind to IKKβ to block IKKβ phosphorylation to inhibit NF-κB activity. In this study we show that the MC159 protein inhibited three distinct pathways that result in NF-κB activation. MC159 also co-immunoprecipitated with the IKK complex, nor did MC159 require IKKα or IKKβ to co-immunoprecipitate with IKKγ. We also observed MC159-IKKγ interactions in either MCV-infected cells or cells in which MC159 was expressed by a surrogate virus [40].

The upstream signal transduction pathways triggered by TNF, MyD88 and PMA converge upon IKK. Since MC159 inhibited NF-κB triggered through each of these

effectors, we speculated that MC159 targeted the IKK complex as its mechanism. Our current model is that the MC159 protein interacts directly with IKKγ to inhibit NF-κB activation. This model is based on our data that show the absence of IKKγ prevents MC159-IKKβ interactions. IKKγ is predicted to have different conformations in its resting versus active states, with the former a compact, coiled protein and the latter a fully extended protein with alpha helices. Whether MC159 preferentially interacts with either form is unknown. If MC159 interacted with only the coiled form, then MC159-IKKγ interactions would prevent IKKβ phosphorylation. It is also known that IKKγ must be post-translationally modified (ubiquitination or phosphorylation) for activity [15]. Thus, another possibility is MC159 may be outcompeting another cellular IKKγ binding protein, preventing one of the previously mentioned post-translational modification events from occurring. Regardless of the mechanism, we propose that inhibition of the MC159 protein would hamper the ability of MCV to survive and replicate in keratinocytes.

We showed previously that MC159 inhibited TNF-induced NF-κB activation. In that publication, it was noticed that MC159 expression itself stimulated low levels of NF-κB-controlled luciferase activity. We saw a similar trend here with both wild-type and mutant MC159 proteins. In agreement with our findings, a recent study reports that the MC159 protein can function as an activator of NF-κB [4]. It was proposed that the MC159 protein, when expressed at low levels, activates NF-κB by enhancing interactions between TNF-R1 signalsome components, including TRAF2. In this study, we found no correlation between the ability of a mutant MC159 protein to bind to TRAF2 and to either stimulate NF-κB activation autonomously or inhibit TNF-induced NF-κB

activation. Recent studies show that TRAF2 interacts with the IKK complex. Whether MC159-TRAF2 interactions would contribute to the stability of MC159-IKKγ interactions, and inhibition of NF-κB, is currently under investigation.

Analysis of the MC159 structure revealed that its tandem DEDs associate with one another in a ridged dumbbell-like structure [28, 47]. Structure-based sequence analysis of the MC159 DEDs shows similarities with other DED-containing proteins (i.e.; c-FLIP, procaspase-8, the HHV8 K13 protein). A striking difference between MC159 and K13 is that MC159 inhibits NF-κB activation whilst K13 stimulates it. Recently, Bagneris et al. co-crystallized K13 with IKKγ, showing that the N-terminal DED of K13 possesses two clefts for binding IKKγ monomers. Based on a sequence alignment between K13 and MC159, Bagneris et al suggests that the MC159 protein lacks the clefts required for IKKγ interactions, and that this loss of interaction in why the MC159 protein does not activate NF-κB. It is suggested that the residues required for formation of cleft 2 are absent in MC159, and that an extra helix in the N-terminus of MC159 (H0) prevents IKKγ from binding to cleft 1. However the H0 region of MC159 has not been successfully crystallized, making it hard to assess if this occlusion occurs *in vivo*.

The MC159 21 and MC159 24 proteins allowed and inhibited TNF-induced NF-κB activation, respectively. The mutations in these proteins do not occur in the MC159 regions that Bagneris predicted to interfere with IKKγ binding. Attempts to co-immunoprecipitate each mutant protein with IKKγ rendered irreproducible results. Interestingly, previous studies of the K13 protein found residues within the N-terminal RXDL motif, a region that is conserved with MC159 and required for NF-κB inhibition, were required for K13's function despite not being required for IKKγ binding. These data

suggest that the biological properties of K13 and MC159 are not yet fully understood. Interestingly, despite having different effects on NF-κB activity, MC159 and K13 both utilize residues within the RXDL motif of DEDA to manipulate NF-κB signaling, suggesting that seemingly homologous viral proteins may evolve to provide different mechanisms that enable virus survival and pathogenesis.

Motifs within the MC159 DEDs that are predicted to mediate protein-protein interactions include a surface-exposed charged region called the RXDL motif. Earlier studies revealed that the RXDL motifs in both DEDA and DEDB must be present in MC159 to inhibit TNF-induced apoptosis [13]. Surprisingly, only the RXDL motif of DEDA was required to inhibit TNF-induced NF-κB activation [34]. This suggests the MC159 protein uses different molecular mechanisms to inhibit apoptosis and NF-κB. TNF is a potent pro-inflammatory cytokine that initiates NF-κB activation or apoptosis via distinct signal transduction pathways (Figure 2.9). It remains unclear how TNF-R1 dictates the activation of either apoptosis or NF-κB [5, 21]. If this information were known, novel therapeutics could be rationally designed to precisely inhibit unwanted or enhance desired TNF-R1 signaling. Since the MC159 protein can inhibit both outcomes of TNF-R1 signaling, through distinct mechanisms, the study of MC159 can provide unique insights into how TNF-R1 initiates either apoptosis or NF-κB activation [20, 34].

TNF-induced NF-κB activation is a biphasic event [19, 37, 42]. The IκBα protein is degraded during acute TNF activation, while the IκBβ protein is degraded after prolonged TNF signaling. Since MCV infections are persistent, it is likely that both acute and chronic TNF-induced NF-κB activation occurs in MCV-infected cells *in vivo*. Interestingly, it is known that MC159 inhibits degradation of IκBβ where MC160 inhibits

degradation of IκBα [34, 35]. Our current working model is that MC160 inhibits acute TNF-induced NF-κB activation that would occur during initial infection, and MC159 inhibits chronic TNF-induced NF-κB activation that would occur over the course of a persistent infection (Figure 2.9). We also hypothesize that the complete silencing of TNF signaling via co-expression of MC159 and MC160 is critical for MCV to sustain a persistent infection while minimizing inflammatory responses. Unfortunately, this hypothesis is difficult to test because of a lack of animal models for MCV infections.

Recent reports have shown that IKK is constitutively active in many cancers, including melanomas [10, 22, 30]. For melanomas, silencing IKK activation results in their apoptotic cell death. In these studies, IKK activation was inhibited by treatment of cells with a NEMO binding peptide (NBP), which binds to IKKγ to disrupt the IKK complex [22, 30]. The MC159 protein is potentially a more useful therapeutic than NBP because it inhibits NF-κB activation without disrupting the IKK complex, and therefore should be less toxic. Additionally, data from a clinical case report empirically indicate that MCV infection has some anti-cancer properties [11]; a patient developed an MC lesion within a cutaneous melanoma, and histological analysis revealed the destruction of melanoma cells at the site of MC lesion. Such therapeutic potential of MC159 needs to be pursued.

5) Figures

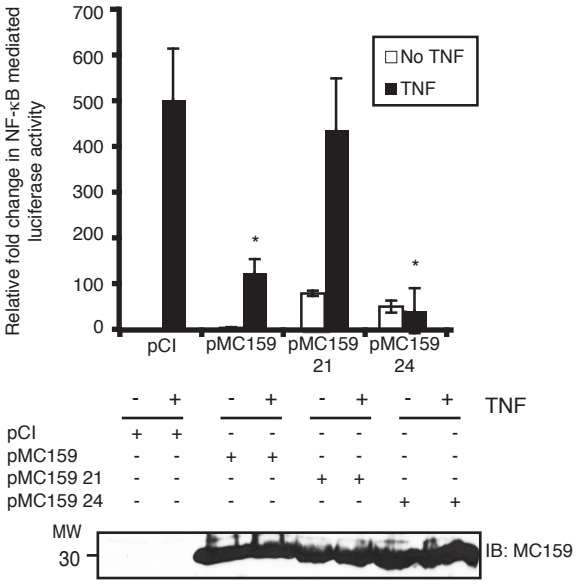

Figure 2.1 The MC159 RXDL motif confers resistance to TNF-induced NF-κB activation. Subconfluent 293T cellular monolayers were transfected with pNF-κBluc, pRL-null, and either pCI, pMC159, pMC159 21 or pMC159 24. At 24 h later, cells were incubated with regular medium or medium containing TNF (10 ng/ml). After an additional 12 h incubation, cells were lysed and luciferase activities were measured. Statistically significant data for MC159 inhibition of NF-κB-mediated luciferase activity ($p<0.05$) are indicated with an asterisk. Lysates also were analyzed by immunoblotting. Proteins were subjected to SDS-12% PAGE, transferred to PVDF membranes and incubated with an anti-MC159 antiserum.

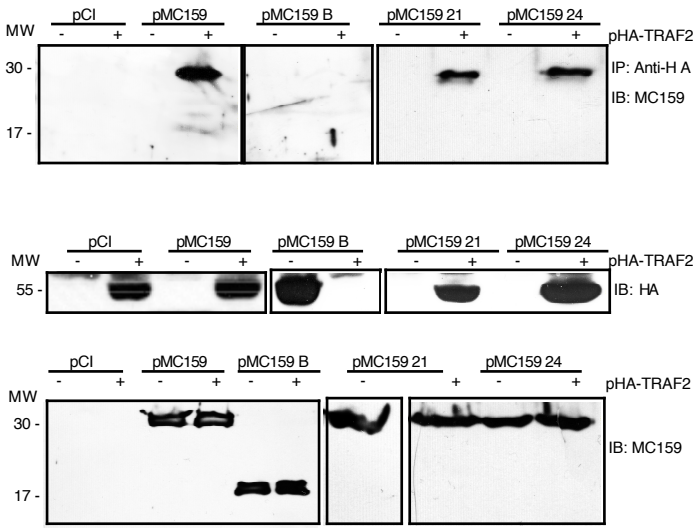

Figure 2.2 TRAF2-MC159 interactions are insufficient to inhibit NF-κB. Subconfluent 293T cellular monolayers were transfected with pHA-TRAF2 and either pCI, pMC159, pMC159 B, pMC159 21 or pMC159 24. At 24 h post-transfection, cytoplasmic extracts were prepared using DED lysis buffer. Immunoprecipitations (IP) were performed by incubating clarified cellular lysates with anti-HA antiserum and protein G-Sepharose beads. Immunoprecipitates (top) or corresponding clarified cellular lysates (middle and bottom panels) were subjected to SDS-12% PAGE. The separated proteins were transferred to PVDF membranes, and immunoblotted (IB) with either anti-HA or anti-MC159 antibodies.

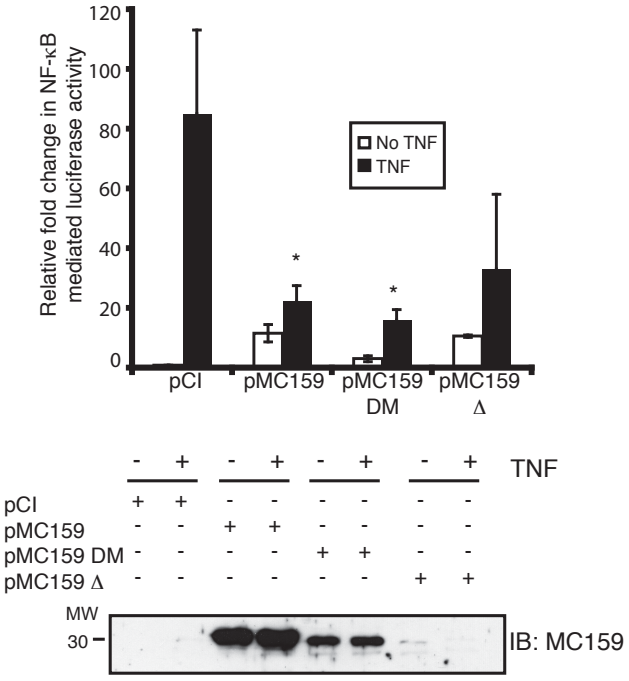

Figure 2.3 The MC159 protein inhibits NF-κB activation independent of TRAF3. Subconfluent 293T cellular monolayers were transfected with pNF-κBluc, pRL-null, and either pCI, pMC159, pMC159 DM or pMC159 Δ. At 24 h later, transfected cells were incubated with regular medium or medium containing TNF (10 ng/ml) for 12 h, after which time cells were lysed and luciferase activities were measured. Statistically significant data sets for MC159 inhibition of NF-κB-mediated luciferase activity ($p<0.05$) are indicated with an asterisk. Lysates also were analyzed for MC159 proteins by immunoblotting. Following SDS-12% PAGE, proteins were transferred to PVDF membranes and incubated with an anti-MC159 antibody.

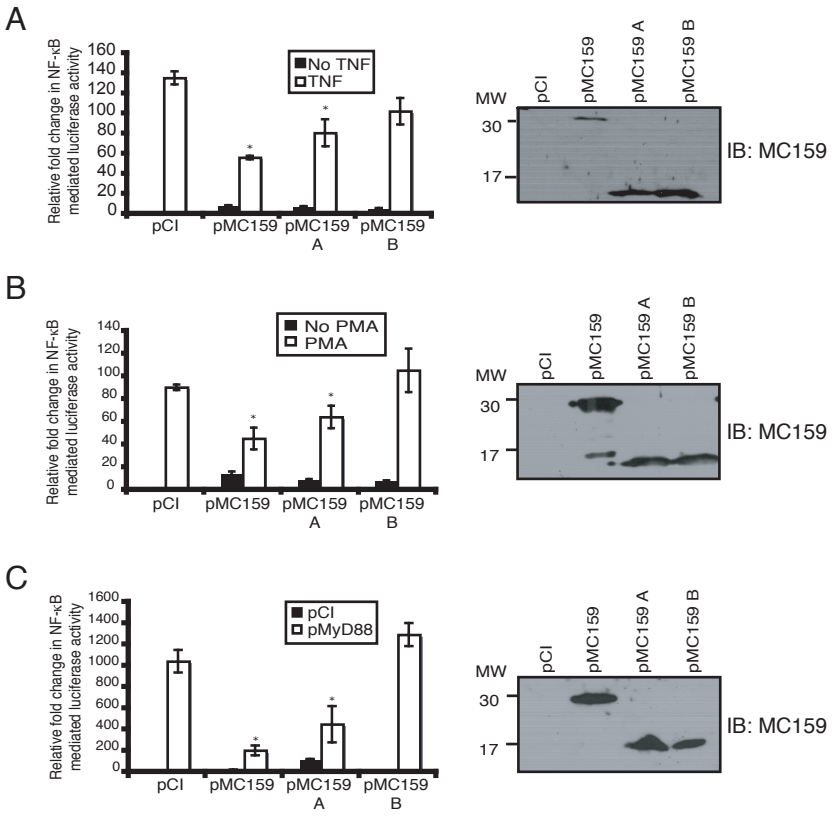

Figure 2.4 The MC159 DEDA inhibits TNF-, PMA- or MyD88- induced NF-κB activation. Subconfluent 293T cellular monolayers cells were transfected with pNF-κBluc, pRL-null, and either pCI, pMC159, pMC159 A or pMC159 B. Transfected cells were incubated with regular medium or medium containing (A) TNF (10 ng/ml) for 8 h or (B) or PMA (50 ng/ml) for 8 h. (C) Subconfluent 293T cell monolayers were transfected with pNF-κBluc, pRL-null, pMyD88 and either pCI, pMC159, pMC159 A or pMC159 B for 24 h. For all experiments, cells were lysed using passive lysis buffer and luciferase activities were measured. Statistically significant data sets for MC159 inhibition of NF-κB mediated luciferase activity ($p<0.05$) are indicated with an asterisk. Lysates were analyzed by immunoblotting. Following SDS-12% PAGE, proteins were transferred to PVDF membranes and incubated with an anti-MC159 antibody.

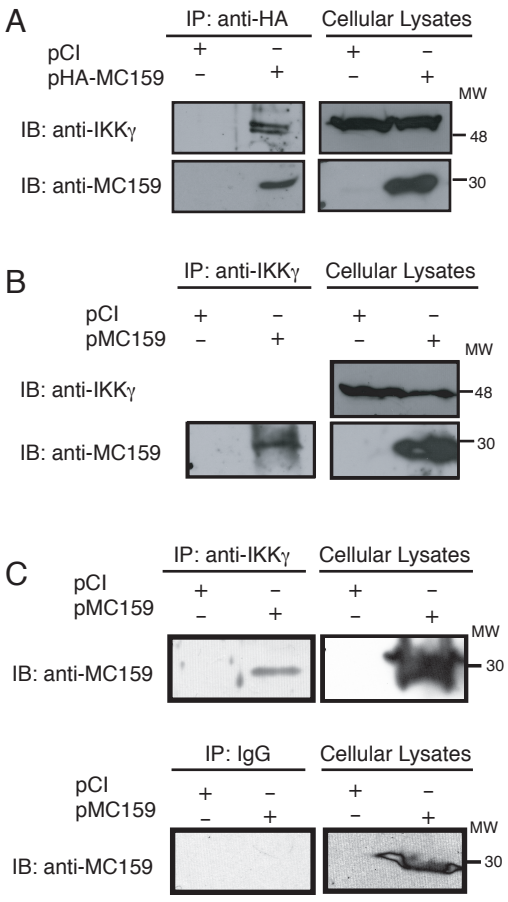

Figure 2.5 The MC159 protein associates with IKKγ. Subconfluent 293T cellular monolayers were transfected with pCI or pHA-MC159. At 24 h post-transfection, cellular lysates were prepared using DED lysis buffer and immunoprecipitations (IP) were performed using protein G-Sepharose beads and either (A) anti-HA or (B) anti-IKKγ antibodies. Immunoprecipitated samples (left panels) or clarified cellular lysates (right panels) were analyzed by immunoblotting. Following SDS-12% PAGE, proteins were transferred to PVDF membranes, and incubated with either mouse anti-IKKγ or anti-MC159 antibodies.

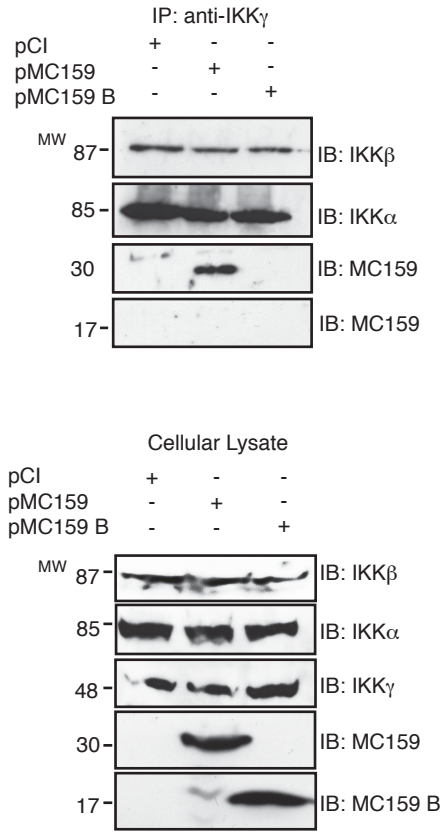

Figure 2.6 IKKγ co-immunoprecipitates IKKα and IKKβ in the presence of MC159 protein. A 10-cm^2 dish of subconfluent MEF cellular monolayers were transfected with pCI, pMC159 or pMC159 B. At 24 h post-transfection cells were lysed in 150 μl DED lysis buffer. 50 μl of clarified lysates were collected to new tubes, and 30 μg of this sample were analyzed for MC159 and IKK proteins by immunoblotting (bottom panel). Immunoprecipitations (IP) were performed using 100 μl clarified lysates incubated with protein G-Sepharose beads and anti-IKKγ. Immunoprecipitated samples (top panel) or clarified cellular lysates (bottom panel) were analyzed by immunoblotting. Following SDS-12% PAGE, proteins were transferred to PVDF membranes, and incubated with either mouse anti-IKKγ, anti-IKKα, anti-IKKβ or anti-MC159 antibodies.

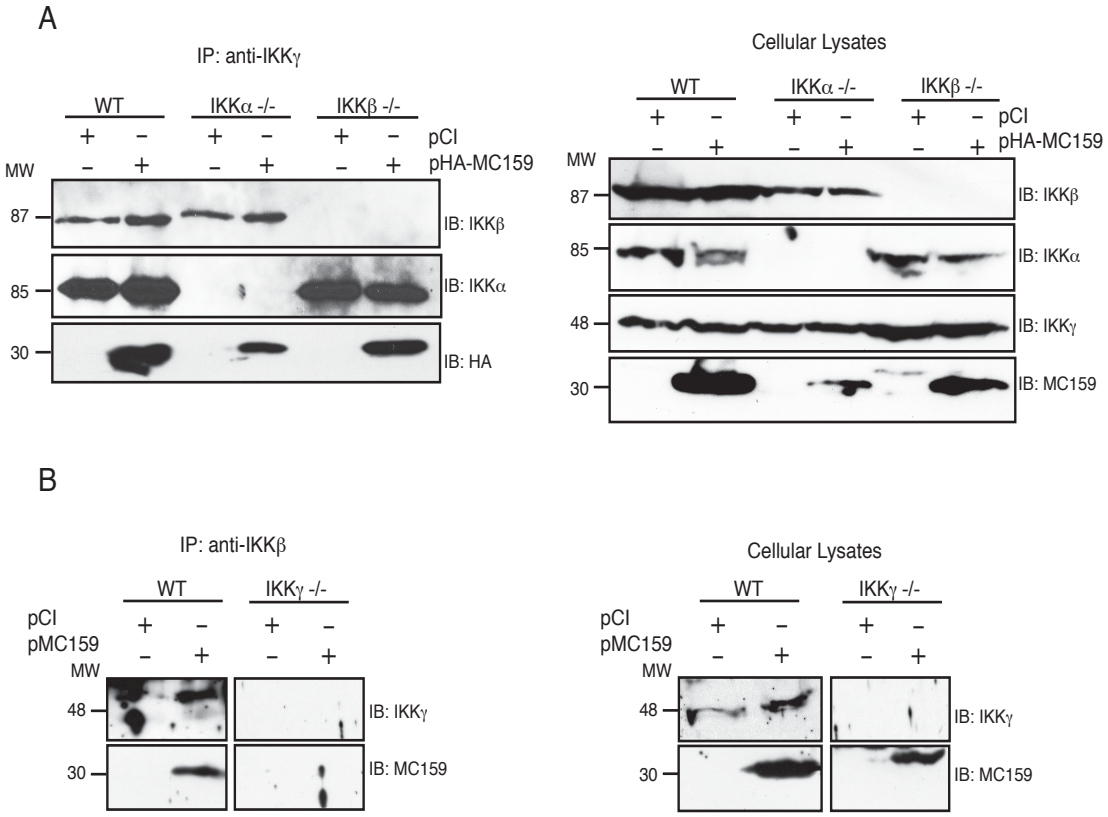

Figure 2.7 The MC159 protein associates with IKKγ independent of IKKα and IKKβ. Subconfluent monolayers of WT, IKKα –/–, or IKKβ –/– MEF cells were transfected with pCI or pHA-MC159. At 24 h post-transfection cellular lysates were prepared using DED lysis buffer, and immunoprecipitations (IP) were performed, by incubating clarified cellular lysates with protein G-Sepharose beads and an anti-IKKγ antibody. Immunoprecipitated samples (top panels) or clarified cellular lysates (bottom panels) were analyzed by immunoblot. Following SDS-12% PAGE, proteins were transferred to PVDF membranes; incubated with either mouse anti-IKKγ, anti-IKKα, anti-IKKβ, anti-HA or anti-MC159 antibodies.

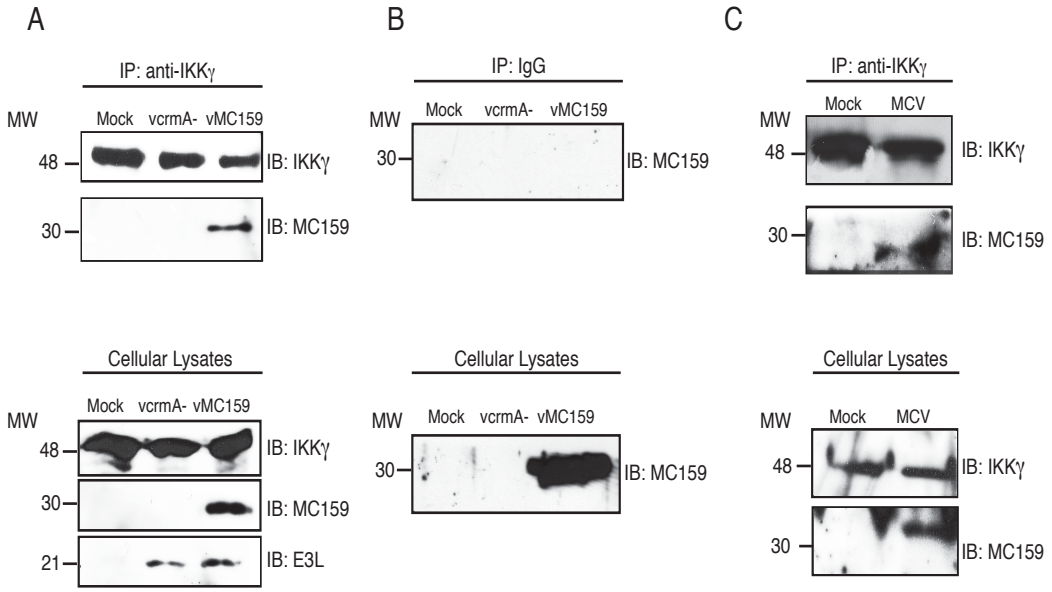

Figure 2.8 The MC159 protein associates with IKKγ during poxvirus infection. (A) Subconfluent 293T cellular monolayers were either mock infected or infected with vcrmA- or vMC159 (MOI = 10). (B) Subconfluent MRC-5 cell monolayers were mock-infected or with 300 μl MCV lesion preparation. For all experiments, at 24 h post-infection cellular lysates were prepared using DED lysis buffer and immunoprecipitations (IP) were performed on clarified lysates using protein G-Sepharose beads and anti-IKKγ antiserum. Immunoprecipitated samples (top panels) or clarified cellular lysates (bottom panels) were analyzed by immunoblot. Following SDS-12% PAGE, proteins were transferred to PVDF membranes, and incubated with rabbit anti-IKKγ, anti-MC159 or anti-E3 antibodies.

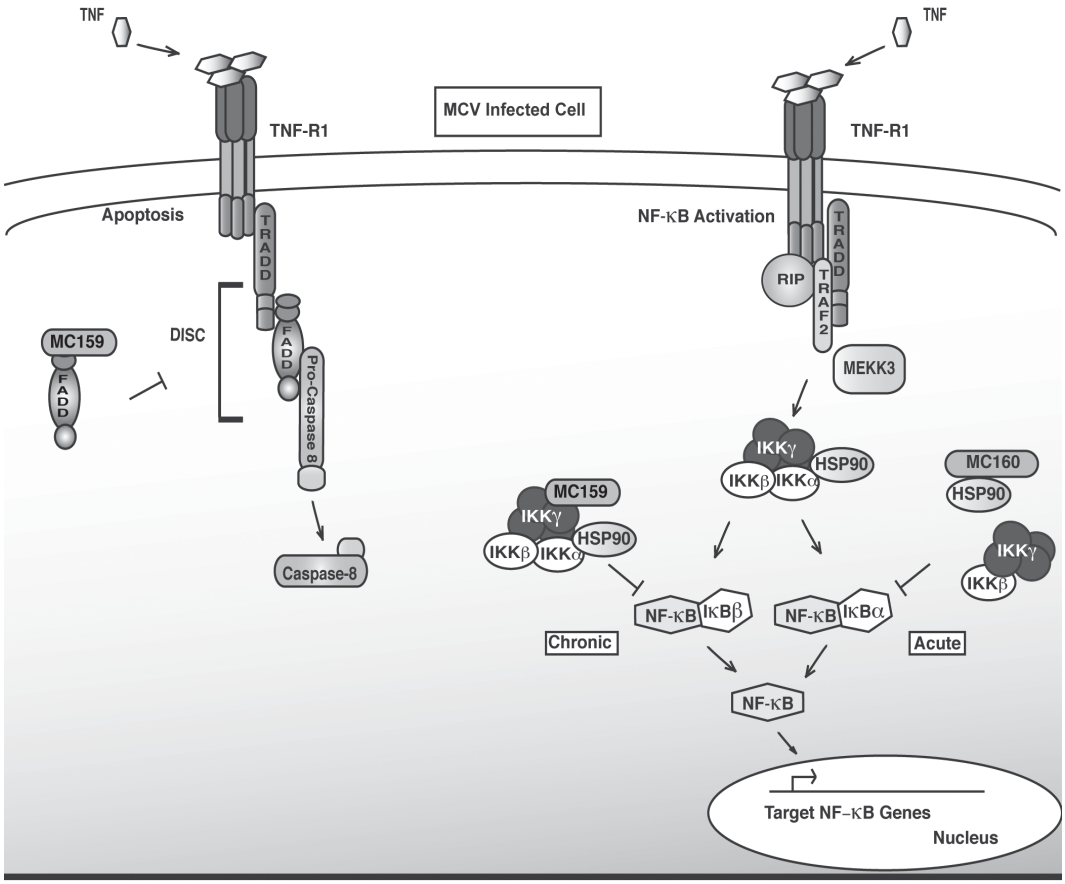

Figure 2.9: A model of MC159 and MC160 inhibition of TNF-R1 signaling during TNF binds to the TNF-R1 receptor to initiate either apoptosis or NF-κB activation. For apoptosis, TNF-TNF-R1 interactions initiate recruitment and activation of the death inducing signaling complex (DISC), composed of TRADD, FADD and procaspase-8. The MC159 protein binds to FADD and prevents DISC assembly to inhibit TNF-induced apoptosis during MCV infection. NF-κB activation is initiated by TNF binding TNF-R1, which recruits TRADD, TRAF2, RIP and MEKK, resulting in the IKK complex activation and the degradation of IκB proteins thus enabling NF-κB activation. We propose the following model for MC159- and MC160- mediated inhibition of TNF signaling during MCV infection: During the initial stage of an MCV infection, the MC160 protein inhibits acute NF-κB activation by binding to Hsp90 to cause IKKα degradation, subsequently preventing the degradation of IκBα (51). Later during MCV infection, the MC159 protein inhibits chronic NF-κB activation by binding to the IKKγ subunit of the IKK complex, thereby inhibiting IκBβ degradation.

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Chapter 3: The MCV MC159 protein inhibits IFN-β expression by preventing phosphorylation of TANK-Binding Kinase-1

1) Introduction

Interferon-beta (IFN-β) provides an important defense against viral infections [31, 41, 42]. The pathway triggering IFN-β production is regulated by a series of signaling cascades. By-products of viral infection first stimulate IFN-β production, resulting in IFN-β secretion and binding to the IFN receptor (IFNR). This initiates a second signaling cascade in infected and neighboring cells, which promotes expression of interferon stimulated genes (ISGs) whose products contribute to an anti-viral state.

IFN-β gene expression is induced when host cell pattern recognition receptors (PRRs) detect pathogen associated molecular patterns (PAMPs). For example, the retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated factor gene 5 (MDA5) PRRs detect viral RNAs [17, 20, 39, 57, 58]. RIG-I and MDA-5 interact with mitochondrial anti-viral signaling adaptor protein (MAVS) via CARD domains [21, 22, 46]. The TNF receptor associated factor (TRAF3) adaptor protein is recruited, followed by the activation of the kinase complex TANK binding kinase-1 (TBK-1): IκB kinase ε (IKKε) [4, 8, 36, 47]. TBK-1:IKKε phosphorylates and activates the IFN regulatory factors, IRF3 and IRF7 [8], which migrate to the nucleus to bind to the IFN-β enhancer. The IFN-β enhancer contains four Positive Regulatory Domains (PRD I-IV): IRF3 and IRF7 bind PRDI/III, NF-κB binds PRDII and AP-1 binds PRDIV [27, 38].

Molluscum contagiosum virus (MCV) is a dermatotropic poxvirus that causes benign skin neoplasms in humans. There is no cure for an MCV infection, and infections

can persist in children and immunocompromised patients [3, 23, 53, 55]. Like other poxviruses, MCV possesses myriad genes that encode immune evasion proteins, and the study of these proteins may uncover mechanisms underlying persistent MCV infections and potential cures [30, 45]. With the eradication of smallpox, MCV is the only poxvirus that has a strict human host range. Regrettably, no animal or tissue culture models exist for MCV infection, making it difficult to assess the effect of these immunomodulatory proteins on the virulence or persistence of MCV infections.

The MC159 protein is an important immunomodulatory protein of MCV and is incompletely characterized. It is a member of the FLIP family of proteins, which includes other viral and host cellular proteins [44, 45]. MC159 is known to inhibit apoptosis by binding to FADD to disrupt the apoptosis-inducing DISC complex [9, 10, 25], and to bind to IKKγ (also referred to as NEMO) to prevent NF-κB activation [40]. Another function ascribed to MC159 is preventing IFN-β enhancer activation[2]. It is well-known that the IFN-β enhancer contains NF-κB binding sites [27, 38]. It remains unclear whether the MC159 IFN-β inhibitory function is an indirect consequence of one of the known MC159 activities e.g., its apoptosis or NF-κB inhibitory functions or if it represents a novel direct activity of MC159[2, 9, 10, 25, 32, 40]. To fill this gap in knowledge and elucidate the mechanism of MC159 regulation of IFN-β production, wild-type MC159 and mutant forms defective for inhibiting NF-κB activation or apoptosis were tested for regulation of the IFN-β response. Data presented here suggest that MC159 inhibits IFN-β enhancer activation independent of its NF-κB inhibitory function, by acting at the level of TBK-1 activation to inhibit IRF3 activation. Furthermore, we

show that the MCV MC160 and cFLIP$_L$ proteins also inhibit MAVS-induced activation of the IFN-β enhancer, suggesting a third, bona fide function of DED motifs.

2) Materials and methods

Cell culture and plasmids.

Human embryonic kidney 293T (293T) cells and human cervical cancer cells (HeLa) were obtained from the American Type Culture Collection. Wild-type mouse embryonic fibroblast (MEF) cells were obtained from Dr. Michael May (University of Pennsylvania). P65 -/- MEFs were obtained from Dr. Laurent Poliquin (University of Quebec). All cells were cultured in Eagle's MEM supplemented with 2 mM L-glutamine, 10% fetal calf section, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Plasmid pMC159 consists of the MCV MC159 gene inserted into vector pCI [49]. Plasmid pMC159A encodes a MC159 gene that lacks the C-terminal DED (DED B; residues 95-178) [9]. Plasmid pMC159B encodes a MC159 gene that lacks the N-terminal DED (DED A; residues 7-81) [9]. Dr. Margot Thome (University of Lausanne) provided plasmid pMC159 Δ, which encodes a VSV epitope-tagged MC159 protein in which the TRAF3 binding sites (residues 211-241) are deleted [51]. Plasmid MC160/pCI plasmid contains the MCV MC160 gene [49]. The pK13 plasmid encodes a FLAG tagged K13 gene from Kaposi's Sarcoma Herpes Virus (KSHV) and a the pcFLIP$_L$ plasmid encoding a FLAG tagged human cFLIP$_L$ gene were provided by Dr. Jeffery Cohen (NIH). Dr. Dongwan Yoo (University of Illinois) provided pnsp11, which encodes a FLAG-tagged nsp11 protein from the porcine respiratory virus, and was used as a

control for IFN-β inhibition assays [50]. Dr. Yoo also provided pMAVS, which encodes a FLAG-tagged MAVS protein, and pIRF3, which encodes a FLAG-tagged IRF3 protein. pTBK-1, which encodes a FLAG epitope-tagged TBK-1 protein, was a kind gift from Dr. Siddharth Balachandran (Fox Chase Cancer Center). The following plasmids were used for reporter assays: pRL-null (Promega), pIFN-β–luc, and pIRF3-luc (gifts from Dr. Dongwan Yoo). For all experiments involving plasmids, DNA was transfected into cells using TransIT-2020 transfection reagent (Mirusbio), following the manufacturer's protocol.

Reverse transcriptase (RT) PCR.

Subconfluent 293T cellular monolayers were co-transfected with 500 ng pCI or pMAVS and 1000 ng pCI, pMC159 or pnsp11. At 24 h post-transfection, total cellular RNA was harvested using the Qiagen RNeasy kit per manufacturer's instructions. A portion of total RNA (500 ng) was reverse transcribed using Mul-V reverse transcriptase (NEB) and oligo d(T) primers (Integrated DNA Technologies). Next, a portion of the cDNA sample (10 μl) was subjected to PCR amplification, using primers specific for either human IFN-β (5'-GCTCTCCTGTTGTGCTTCTCCACTACAGC-3' and 5'-CTGACTATGGTCCAGGCACAGTGACTGTACTCC-3') [1] or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)(5'-AAGGTCGGAGTCAACGGATTTGGT-3' and 5'-ACAAAGTGGTCGTTGAGGGCAAT-3'). PCR conditions were 95°C for 30 s, 62°C for 45 s, and 72°C for 30 s for 25 cycles. An aliquot of each PCR reaction was subjected to agarose gel electrophoresis, and amplicons were visualized by ethidium bromide staining of gels.

Luciferase Assays.

Subconfluent MEFs, p65 -/- MEFs or HeLa cellular monolayers in 12-well plates were co-transfected with 25 ng pRL-null, 225 ng IFN-β-luc, 500 ng pCI or pMAVS and 750 ng of either pCI, pMC159, pMC159A, pMC159B, pMC160, pcFLIP$_L$, pK13 or pnsp11. At 24 h post-transfection, cells were lysed in 1X passive lysis buffer (PLB; Promega), and lysates were analyzed for firefly and sea pansy luciferase activities. In another experiment in which poly (I:C was used to activate the MAVS pathway, subconfluent HeLa cell monolayers were co-transfected with 225 ng pIRF3-luc, 25 ng pRL-null, and 750 ng of either pCI, pMC159, pMC159A, or pMC159B, pMC159Δ or pnsp11. At 24 h post-transfection, cells were transfected with 500 ng poly (I:C) (Sigma Aldrich). At 18 h later, cells were lysed in 1X PLB and analyzed for luciferase activities. In another luciferase reporter assay, over-expression of TBK-1 was used to activate IRF3. In this case, subconfluent 293T cells were co-transfected with 225 ng pIRF3-luc, 25 ng pRL-null, 500 ng pTBK-1 or pCI, and 750 ng of either pCI, pMC159, pMC159A, pMC159B, pMC159Δ or pnsp11. At 48 h later, cells were lysed in 1X PLB, and lysates were analyzed for luciferase activities.

All luciferase activities were measured as relative light units using a BioTek luminometer and the Dual Luciferase Reporter Assay System (Promega). All experiments were performed in triplicate. Relative luciferase activity ratios were calculated by dividing firefly luciferase activities by sea pansy luciferase activities. Luciferase activity ratios were then normalized to that of pCI-transfected cells incubated in regular medium,

and this value was set as 1. Statistical significance was calculated using a Student t test where $p < 0.05$. A portion of each cellular lysate from luciferase assays was analyzed for protein expression by immunoblotting (see below).

Immunoblotting and Antibodies.

The activation states of IRF3 or TBK-1 were determined by detecting the phosphorylated (activated) forms of each protein by using immunoblotting. To detect phospho-IRF3, subconfluent HeLa cells were co-transfected with 500 ng of pIRF3 and 1000 ng of either pCI, MC159, pMC159A, pMC159B, pMC159Δ or pnsp11. At 24 h later, cells were transfected with 2000 ng poly (I:C). At 18 h later, cells were lysed in cytoplasmic extraction buffer [37]. To detect IRF3 proteins, HeLa cells were co-transfected with 300 ng pIRF3 or pCI and 1000 ng pMC159, pMC159A, pMC159B, pMC159Δ or pnsp11. At 24 h later, cells were lysed in CE buffer. To detect TBK-1 proteins, 293T cells were co-transfected with 500 ng pTBK-1 or pCI and 1000 ng pMC159, pMC159A, pMC159B, pMC159Δ or pnsp11. At 24 h later, cells were lysed in CE buffer.

The protein concentration of each clarified cellular lysate was determined using bicinchoninic acid assay (Pierce). Either 10 or 20 μg (see figure legends) of protein from each lysate was prepared for immunoblotting by suspending in non-reducing lane marker sample buffer (Thermo Scientific) with 5% 2-ME and boiling for 5 min. Clarified cellular lysates were subjected to SDS-10% PAGE, and proteins subsequently were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore), and blocked in TBST containing 5% milk or BSA (phospho-antibodies). Membranes were incubated

with the indicated primary antibodies overnight at 4^{o}C. Next membranes were washed three times in TBST. Then membranes were incubated with the appropriate HRP-conjugated secondary antibody. After three more washes in TBST, immunoblots were incubated with Pierce super signal west chemilluminescence reagents, and blots were exposed to x-ray films (Fisher Scientific).

Primary antibodies used for these experiments were: polyclonal rabbit anti-MC159 (1:1000), monoclonal mouse anti-FLAG (1:500; Sigma-Aldrich), polyclonal rabbit anti-phospho-TBK1 (Ser 172) (1:500; Millipore), monoclonal rabbit TBK-1 (1:1000 cell signaling), monoclonal rabbit (Ser 396) anti-phospho-IRF3 (1:1000; Cell Signaling), monoclonal rabbit anti-IRF3 (1:1000), monoclonal mouse anti-VSV (1:50,000; Sigma-Aldrich) and polyclonal rabbit anti-actin (1:5000; Calbiochem). Secondary antibodies that were used were either HRP-conjugated goat anti-mouse IgG (1:20,000; Thermo Scientific) or HRP-conjugated goat anti-rabbit IgG (1:20,000; Calbiochem).

3) Results

The MC159 protein inhibits IFN-β gene expression.

Recently, it was reported that MC159 inhibits dsRNA-induced activation of the IFN-β enhancer [2]. However, the subcellular mechanism for this inhibition is ill-defined. To fill the gap in this knowledge, a model system was used here, in which the MC159 protein was expressed transiently. As such, MC159 was expressed independent of virus infection, and of other viral proteins that could potentially inhibit IFN-β

activation. Results from two assays confirmed that MC159 negatively impacted IFN-β gene activation. In the first assay, the effect of MC159 was detected by using semi-quantitative RT-PCR to measure IFN-β gene transcription in 293T cells (Figure 1A). This cell line was chosen because of its high transfection efficiency. Transcription of the IFN-β gene was triggered by MAVS over-expression, and transient expression of the MC159 protein greatly reduced MAVS-induced IFN-β gene expression. An IFN-β amplicon was not detected when using a separate set of cells that transiently expressed the PRRSV nsp11 protein, a known inhibitor of IFN-β activation[48]. Levels of GAPDH cDNAs were comparable among all conditions, showing that the diminished amount of IFN-β cDNA was not due to uneven loading of gels with cDNA. A second means to evaluate IFN-β gene activation used a reporter assay, in which the firefly luciferase gene was under the transcriptional control of the natural IFN-β enhancer (Figure 1B). Mouse embryo fibroblast (MEF) cells were transfected with pMAVS to induce IFN-β-enhancer-controlled gene transcription. In comparison to cells transfected with empty vector, MC159 expression decreased IFN-β-enhancer-controlled luciferase activity by 63%. Nsp11 protein expression decreased luciferase activity levels to a greater extent than MC159, completely inhibiting luciferase gene expression.

The MC159 protein inhibits IFN-β gene activation independent of its NF-κB inhibitory function.

The IFN-β enhancer is regulated by the NF-κB, IRF3 and AP-1 transcription factors [43]. Since MC159 inhibits NF-κB activation [40], the luciferase reporter assay also was performed using p65 -/- MEFs to study the activity of MC159 in an NF-κB-

independent manner (Figure 1C). p65 -/- MEFs were less responsive to MAVS stimulation than wild-type MEFs. Despite this difference, we still observed significant activaiton of the IFN-β enhancer when MAVS was overexpressed. Although NF-κB independent IFN-β activation observed in these cells is less robust it representative of the IRF transcription such as, IRF3 which exclusively regulate IFN enhancers. MC159 reduced MAVS-induced luciferase activity by 64% as compared to vector (pCI)-transfected cells, suggesting that MC159 inhibition of IFN-β expression was independent of its NF-κB inhibitory function. As would be expected, IFN-β-enhancer-controlled luciferase activity was diminished in nsp11-expresing cells. Additionally, MC159 and nsp11 proteins were detected in similar levels in unstimulated and stimulated MEF and p65 (-/-) cells (Figures 1B and 1C), showing that MAVS over-expression did not affect the stability of either protein.

The MC159 B protein, which lacks DED A, no longer inhibits TNF-induced NF-κB activation [32, 40]. However, as shown in Figure 2A, the MC159 B protein inhibited MAVS-induced luciferase activity to the same extent as wild-type MC159. The MC159 A protein, which lacks the DED B of MC159, also inhibited IFN-β activation. These data suggested that neither DED of MC159 was required for inhibition of IFN-β enhancer activation. Wild-type and mutant MC159 proteins were expressed similarly under all conditions. Similar effects were observed when identical experiments were performed using p65 (-/-) MEFs (Figure 2B) as a further indication that the MC159 IFN-β inhibitory function was independent of its NF-κB inhibitory function. In both experiments, nsp11 co-expression inhibited MAVS-induced IFN-β-enhancer-controlled luciferase activity to the greatest extent, as expected.

The MC159 protein inhibits IRF3 activation.

IRF3 activation is one of the final downstream events involved in IFN-β gene activation [12, 15]. To test the effect of MC159 on IRF3 activation, a luciferase reporter assay was used in which the firefly luciferase gene was controlled by an IRF3 promoter. In this experimental system, HeLa cells were incubated with poly (I:C), using conditions previously reported to stimulate MAVS and IRF3 activation (Figure 3) [58]. It was observed that this method of luciferase activation was not as robust as MAVS over-expression. Nevertheless, IRF3-controlled luciferase activity was reduced by at least 50% when MC159 as compared to vector-transfected cells, and activity was reduced virtually completely when cells expressed nsp11. As a second assessment of IRF3 activation, the effect of MC159 on poly (I:C)-induced IRF3 phosphorylation was also examined (Figure 4A). Phospho-IRF3 proteins only were detected by immunoblotting when HeLa cells were incubated with poly (I:C). The intensity of this band was reduced by approximately 50% when cells expressed MC159, and the band was no longer visible when cells expressed nsp11.

Both the MC159 A and MC159 B proteins inhibited IRF3 activation in the luciferase reporter and IRF3 phosphorylation assays (Figures 3 and 4, respectively). For both assays, MC159 A had a greater inhibitory effect than MC159 B. Moreover, for assays detecting IRF3 phosphorylation, the phospho-form of IRF3 was no longer detectable when cells expressed MC159 A. The differences observed in phospho-IRF3 levels were not ascribed to IRF3 protein stability since IRF3 protein levels remained equal in cells expressing wild-type or mutant MC159 proteins (Figure 4B).

Previous studies have shown that C-terminus of MC159 possesses a TRAF binding motif its C-terminus, and is known to bind to TRAF3 as well as TRAF1 and TRAF2 [51]. TRAF3 is an adaptor molecule required for IFN-β expression [36]. Interestingly, a mutant MC159 protein deficient in TRAF3 binding (MC159 Δ), decreased IRF3-driven luciferase activity by 58% (Figure 3) and also decreased poly (I:C)-induced IRF3 phosphorylation, suggesting that MC159 inhibits IFN-β expression independently of TRAF3.

The MC159 protein inhibits TBK-1 activation.

TBK-1 activation is an event that occurs upstream of IRF3 activation and downstream of MAVS activation [8]. To assess the effect of MC159 on TBK-1 activation, the first approach was to over-express TBK-1 and assess the MC159 effect on IRF3-induced luciferase activity (Figure 5A). The presence of the wild-type MC159 protein reduced luciferase activity by 57% as compared to pCI-transfected cells (Figure 5B). While MC159 B diminished luciferase activity to a slightly greater extent than MC159 A or MC159 Δ, the reduction in luciferase activities was statistically significant for each viral protein. Viral proteins were expressed at similar levels, indicating that TBK-1 over-expression does not de-stabilize MC159 protein expression. As would be expected, nsp11 inhibited TBK-1 induced luciferase activity the greatest. It should also be mentioned that a recent report shows that MC159 activates NF-κB [6]. Under the conditions tested here, the over-expression of MC159 did not induce IFN-β enhancer activity or IRF3 activating reporter.

As a second means to assess TBK-1 activation, the effect of MC159 on TBK-1 phosphorylation was also examined by using immunoblotting. As show in Figure 6A, the over-expression of TBK-1 stimulated TBK-1 phosphorylation in vector-transfected cells. The intensity of the phospho-TBK-1 containing band was greatly diminished when cells expressed wild-type MC159, MC159 B or MC159 Δ. Moreover, phospho-TBK-1 was not detected when cells expressed either MC159 A or nsp11. Actin levels were similar among the all conditions, indicating equal protein loading (Figure 6A). Figure 6B showed that TBK-1 levels were unchanged in MC159-expressing cells suggesting that the MC159 mechanism of inhibition did not rely on TBK-1 degradation.

The MCV MC160 and cellular FLIP$_L$ also inhibit IFN-β-enhancer-controlled luciferase activity.

Other cellular and viral FLIPs, including the MCV MC160 protein, cFLIP$_L$ and the Kaposi Sarcoma Herpes Virus (KSHV) K13 protein, also contain two tandem death effector domains [19, 25, 52, 56]. Since these FLIPs modulate either NF-κB activation or apoptosis we hypothesized that they inhibited IFN-β expression [5, 7, 18, 28, 34, 35]. To this end, we tested whether these FLIPs affected MAVS- induced luciferase activity in p65 -/- MEFs (Figure 7). Similar to Figure 1, MC159 inhibited MAVS-induced IFN-β-enhancer-controlled luciferase activity, and the PRRSV protein nsp11 strongly inhibited luciferase activity. Interestingly both MC160 and cFLIP$_L$ significantly inhibited IFN-β enhancer activation 80% and 87% respectively. Conversely K13 expression only resulted in a 42% decrease in luciferase activity, suggesting K13 is a much weaker inhibitor. Expression of viral proteins was confirmed by immunoblotting, showing that FLIP

proteins were stably expressed under all conditions suggesting differences inhibition among the FLIP proteins was not due to level of protein expression.

4) Discussion

Viruses possess immune evasion mechanisms to aid in their survival against the host immune system. Identifying how the immune evasion proteins function on a subcellular level provides information that may allow for the reconstitution of the immune response to combat or prevent viral diseases. The TBK-1:IKKε complex is an essential complex for activation of IFN-β expression in response to viral infection [8]. Therefore, it is not surprising that TBK-1:IKKε is targeted by viral immune evasion proteins. One such immune evasion protein, the vaccinia virus C6 protein was recently shown to interact with TBK-1:IKKε complex scaffold proteins to block IRF3 and IRF7 controlled IFN-β production [54]. The herpes simplex virus (HSV) γ_1 34.5 protein also prevents TBK-1 activation to block IFN-β expression [26]. Here we describe another viral protein (MC159) that inhibits phosphorylation of the TBK-1. MC159 shares no sequence or structural homology to C6 or γ_1 34.5. As such, it may possess a novel mechanism to control the TBK-1:IKKε complex.

Since DED motifs like those found in MC159 are important for interactions with other binding partners, our leading model is that MC159 binds to TBK-1 to prevent TBK-1 autophosphorylation or association with IKKε. The presence of TRAF3 is also required for TBK-1 activation [36]. While TRAF3:MC159 interactions are known [51], it is unlikely that MC159 competitively binds to TRAF3 to prevent TBK-1 activation because we observed that a mutant MC159 protein which lacks TRAF3 binding motifs

still inhibited TBK-1 activation. Additionally, our data show that K13, which binds to TRAF3[13], only modestly inhibited activation of the IFN-β enhancer. Recently it was reported that the cellular TRAF-interacting protein (TRIP) inhibits TBK-1 by targeting it for ubiquitin-mediated degradation [59]. However, TBK-1 protein levels are unaffected by MC159, suggesting that MC159 is not acting as a TRIP homolog.

A previous study shows that MC159 inhibits IFN-β expression indirectly, by interacting with FADD [2]. It is well-documented that MC159-FADD interactions are necessary for its anti-apoptosis function [25, 56]. However, data shown here suggest that MC159 mediated inhibition of IFN-β expression is independent of FADD interactions and of anti-apoptosis functions; while both MC159 DEDs must be present for inhibition of apoptosis and for FADD interactions [9, 25, 56], neither MC159 DED was required to inhibit IFN-β. In addition, data here show that MC159 inhibits TBK-1 activation, an event that occurs downstream of FADD-induced IFN-β expression.

Several publications show that MC159 inhibits NF-κB activation [11, 32, 40]. This report presents evidence that MC159 inhibits IFN-β in an NF-κB-independent manner. First, MC159 inhibited the expression of an IFN-β enhancer- or IRF3-controlled luciferase gene in cells lacking the p65 subunit of NF-κB. Second, the MC159 B protein, which is a weak inhibitor of NF-κB, inhibits IFN-β enhancer and IRF3-promoter reporter assays to similar levels as wild-type MC159. Although, MC159 A and MC159 B contain different DEDs they both possess the C-terminus, suggesting that neither DED of MC159 was required for inhibition. If MC159 mediated inhibition IFN-β is DED independent, we predict that MC159 is utilizing regions within the C-terminus. However it is possible that either DED of MC159 may be sufficient for inhibition. In this

case each DED may act through a single mechanism, perhaps by targeting TBK-1. Alternatively, each DED may use a distant mechanism. This might explain why MC159 A decreased levels of phospho-IRF3 and phospho-TBK-1 to a greater extent than MC159 B. Further dissection of these regions will be the focus of future studies. We are especially interested in the RXDL motif, given that that motif is necessary for anti-apoptosis and anti-NF-κB functions.

There are three splice variants of cFLIP: $cFLIP_S$, $cFLIP_R$ and $cFLIP_L$. We were particularly interested in studying $cFLIP_L$ because IFN-β protein levels are increased $cFLIP_L$ -/- MEFs, suggesting $cFLIP_L$ inhibits IFN-β expression [14]. Here we show directly that $cFLIP_L$ now has a third function, that of inhibiting IFN-β expression. $CFLIP_L$ is up-regulated in several cancers [24, 29, 33]. Whether this new inhibitory property of $cFLIP_L$ aids in tumor cell survival is unknown. Since the $cFLIP_S$ and $cFLIP_R$ variants also possess tandem DEDs, it is expected that these two forms of cFLIP will also inhibit IFN-β gene activation.

The IRF3 protein is abundant in most cell types and only controls IFN-β expression [43]. Conversely, IRF7 regulates IFN-α and IFN-β [16]. IRF- 7 is expressed at low levels in cells under normal conditions but is induced as a result of continued IFN-β expression. Interestingly, TBK-1:IKKε also regulates IFN-α by activating the IRF7. One possibility, which will be studied in the future, is that MC159 mediated inhibition of TBK-1 inhibits IRF7 activation and IFN-α production.

Here we describe two additional FLIPs, the MCV MC160 and the cellular $cFLIP_L$ that also inhibit the IFN-β enhancer, whereas K13 inhibits to a lesser degree. While all of these FLIPs possess tandem DEDs, they have distinct biochemical properties. For

example, MC159, K13 and $cFLIP_L$ inhibit apoptosis, whereas MC160 does not. Additionally, MC159 and MC160 inhibit NF-κB activation; $cFLIP_L$ activates NF-κB [44, 45]. More curious is that DEDs, although similar, have different phenotypes. Although MC159 requires DED A to inhibit NF-κB activation, MC160 requires DED B [32, 34, 35]. As such, it is unknown whether MC159 and MC160 use similar or distinct mechanisms to inhibit IFN-β expression. Currently it is unclear what differences among these FLIPs allow them to modulate apoptosis, NF-κB activation and IFN-β expression by such different mechanisms. Further study of these FLIPs will lead to great insights into cellular biology and pathogenesis of FLIP producing viruses.

5) Figures

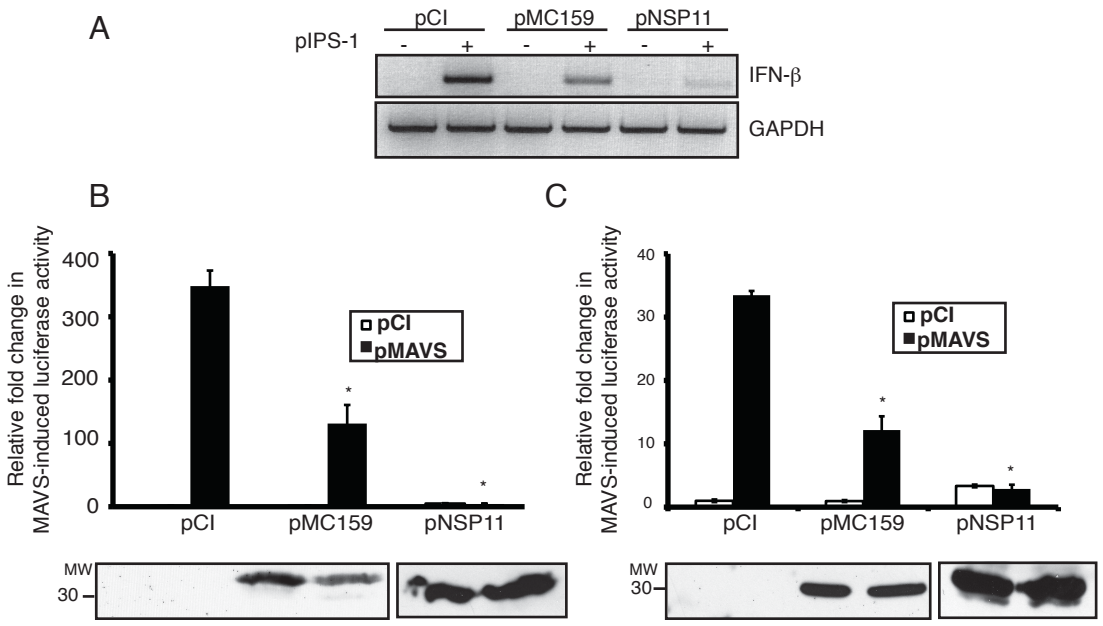

Figure 3.1 The MC159 protein inhibits IFN-β expression. (A) Subconfluent monolayers of 293T cells were transfected with 500 ng of pCI or pMAVS and 1000 ng of pCI, pMC159, or pnsp11. At 24 h post-transfection, cells were lysed and total RNA was isolated from each lysate. RNA was subjected to reverse transcriptase PCR. Lysates were then subjected to RT PCR for IFN-β and GAPDH mRNA. The resulting amplicons were separated by electrophoresis in a 1% agarose gel and visualized with ethidium bromide. Subconfluent (B) WT or (C) p65 -/- MEF cellular monolayers were co-transfected with pIFN-β-luc (225 ng), pRL-null (25 ng), 500 ng pMAVS or pCI, and 750 ng of pCI, pMC159 or pnsp11. Transfections were performed in triplicate. At 24 h later incubation, cells were lysed, and firefly and sea pansy luciferase activities were measured. Statistically significant data for inhibition of IFN-β-mediated luciferase activity ($p<0.05$) are indicated with an asterisk. A portion of each lysate was analyzed for MC159 and nsp11 expression by immunoblotting. Proteins were subjected to SDS-12% PAGE, transferred to PVDF membranes and incubated with an anti-MC159 or anti-FLAG antiserum.

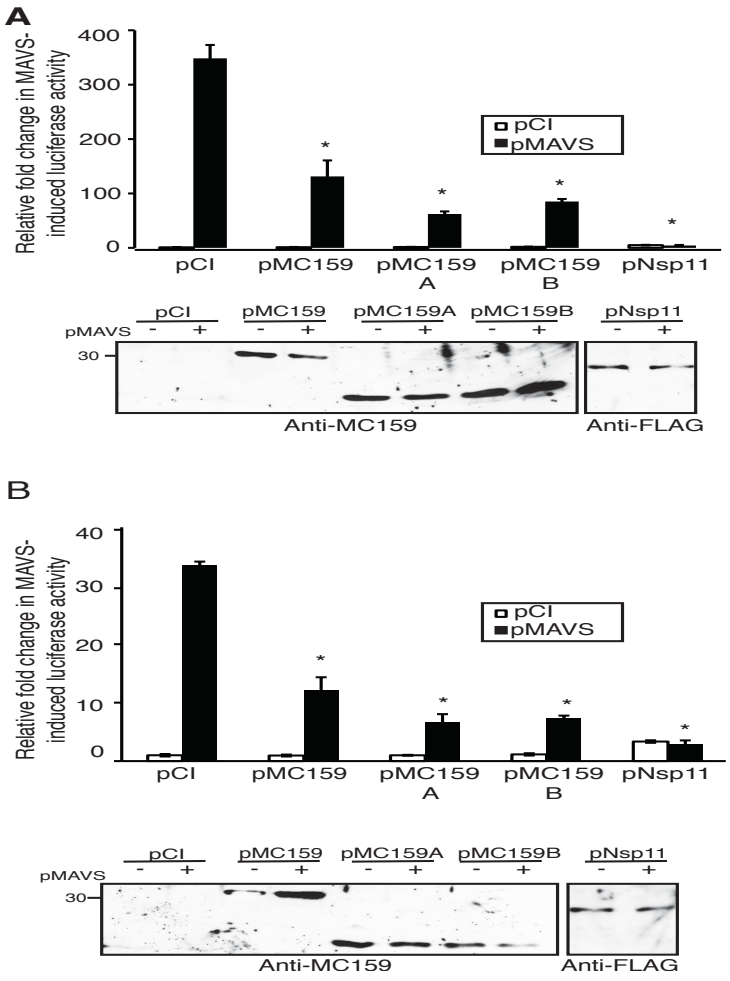

Figure 3.2 Either DED of MC159 is sufficient to inhibit MAVS-induced IFN-β-enhancer-controlled luciferase activity. Subconfluent monolayers of (A) WT or (B) p65 -/- MEFs were co-transfected with pIFN-β-luc (225 ng), pRL-null (25 ng), 500 ng pMAVS or pCI, and 750 ng of pCI, pMC159, pMC159A, pMC159B or pnsp11. Transfections were performed in triplicate. At 24 h later incubation, cells were lysed, and firefly and sea pansy luciferase activities were measured. Statistically significant data for inhibition of IFN-β-mediated luciferase activity ($p<0.05$) are indicated with an asterisk. A portion of each lysate was analyzed by immunoblotting. Proteins were subjected to SDS-12% PAGE, transferred to PVDF membranes and incubated with anti-MC159 or anti-FLAG antiserum.

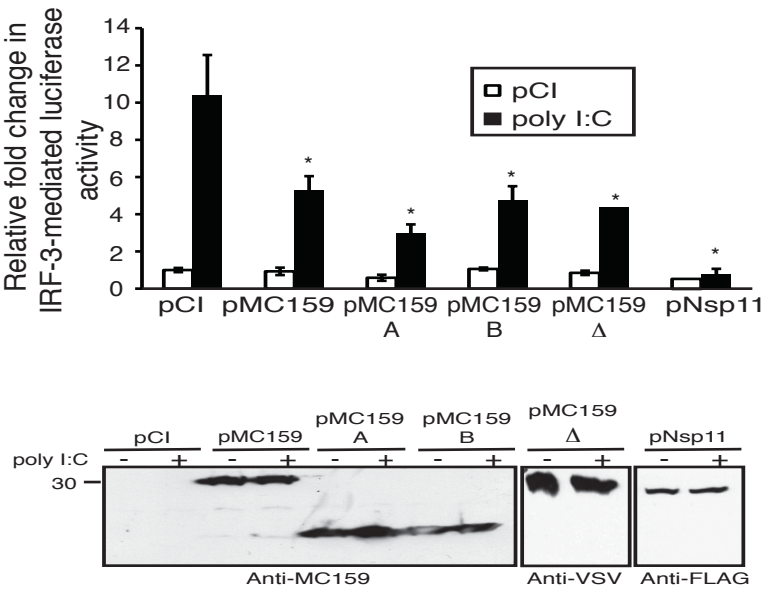

Figure 3.3 The MC159 protein inhibits poly (I:C)-induced IRF3 activation. Subconfluent HeLa cellular monolayers were transfected with pIRF3-luc (225 ng), pRL-null (25 ng), and 750 ng of pCI, pMC159, pMC159A, pMC159B, pMC159Δ or pnsp11. At 24 h later, cells were transfected with 500 ng poly (I:C). Transfections were performed in triplicate. After 18 h incubation, cells were lysed and luciferase activities were measured. Statistically significant data for inhibition of IRF3-mediated luciferase activity ($p<0.05$) are indicated with an asterisk. A portion of each lysate was analyzed by immunoblotting. Proteins were subjected to SDS-12% PAGE, transferred to PVDF membranes and incubated with anti-MC159, anti-VSV or anti-FLAG antiserum.

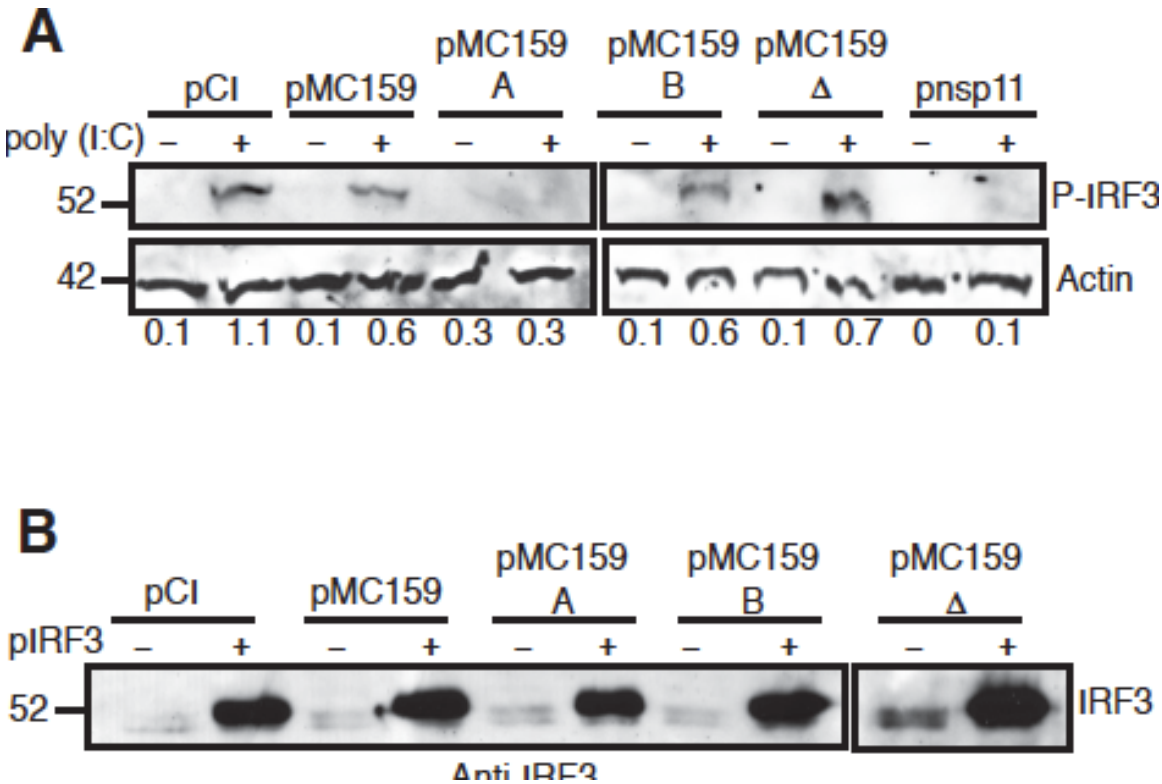

Figure 3.4 The MC159 protein inhibits IRF3 phosphorylation. (A) Subconfluent monolayers of HeLa cells were co-transfected with 300 ng pIRF3 and 1000 ng of either pCI, MC159, pMC159A, pMC159B, pMC159Δ or pnsp11. At 24 h later, cells were transfected with 2000 ng poly (I:C). At 24 h later, cells were lysed, and clarified cellular lysates were analyzed by immunoblotting. (B) Subconfluent of monolayers of HeLa cells were co-transfected with 500 ng of pCI or pIRF3 and 1000 ng of pCI, MC159, pMC159A, pMC159B or pMC159Δ. At 24 h later, cells were lysed, and clarified cellular lysates were analyzed by immunoblotting Following separation by SDS-10% PAGE, proteins were transferred to PVDF membranes, and duplicate blots were incubated with either anti-phospho-IRF3 (P-IRF3), anti-IRF3 or anti-actin antiserum. The intensity of the phospho-IRF3 and actin-containing bands were measured by densitometry, and the ratio of phospho-IRF3 signal to that of actin was determined for each sample, and was plotted below the immunoblot.

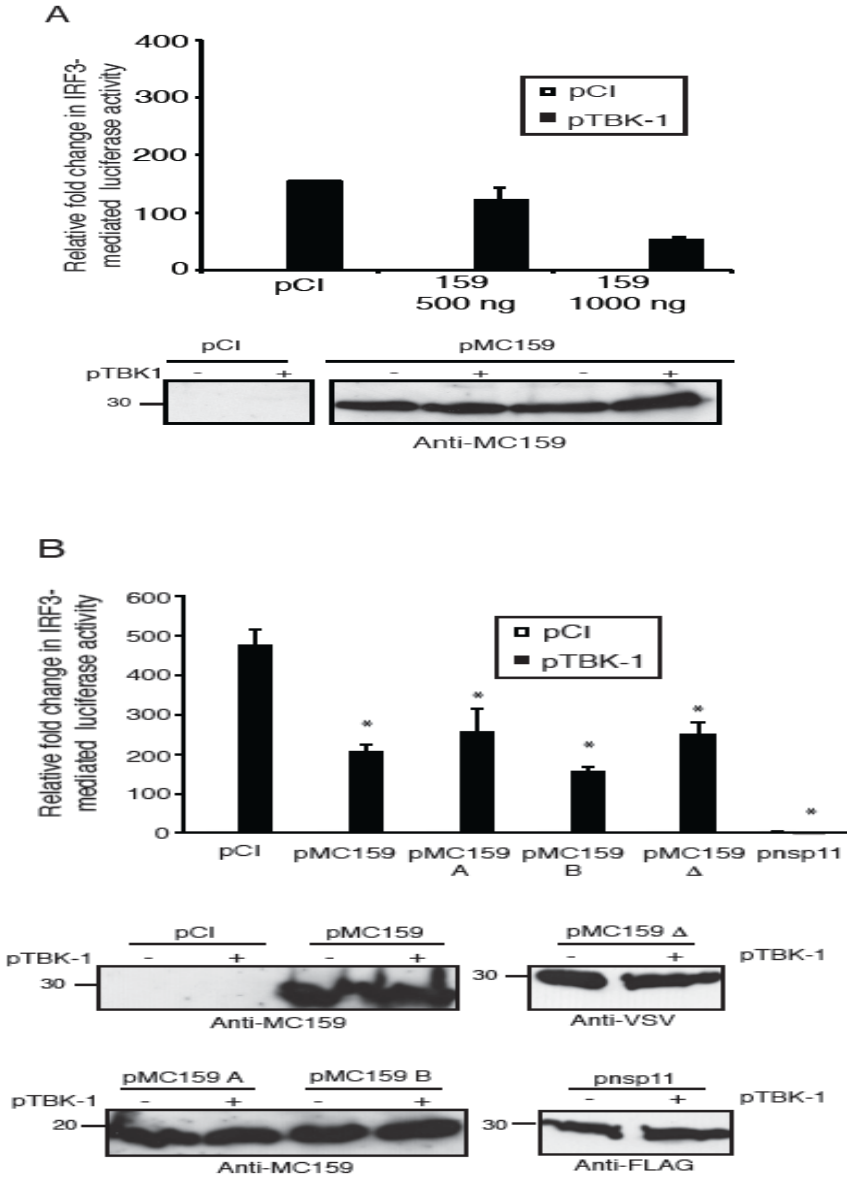

Figure 3.5 The MC159 protein inhibits TBK-1-Induced IRF3 activation. (A) Subconfluent 293T cellular monolayers were transfected with pIRF3-luc (225 ng), pRL-null (25 ng), and 0 ng, 500 ng or 1000 ng of pCI or pMC159. (B) Subconfluent 293T cellular monolayers were transfected with pIRF3-luc (225 ng), pRL-null (25 ng), 500 ng of either pCI or pTBK-1 and 1000 ng of pCI, pMC159, pMC159A, pMC159B, pMC159Δ or pnsp11. Transfections were performed in triplicate. After 48 h incubation, cells were lysed, and firefly and sea pansy luciferase activities were measured. Statistically significant data for MC159 inhibition of IRF3-mediated luciferase activity ($p<0.05$) are indicated with an asterisk. A portion of each lysate was analyzed by immunoblotting. Proteins were subjected to SDS-12% PAGE, transferred to PVDF membranes and incubated with anti-MC159 or anti-VSV antiserum.

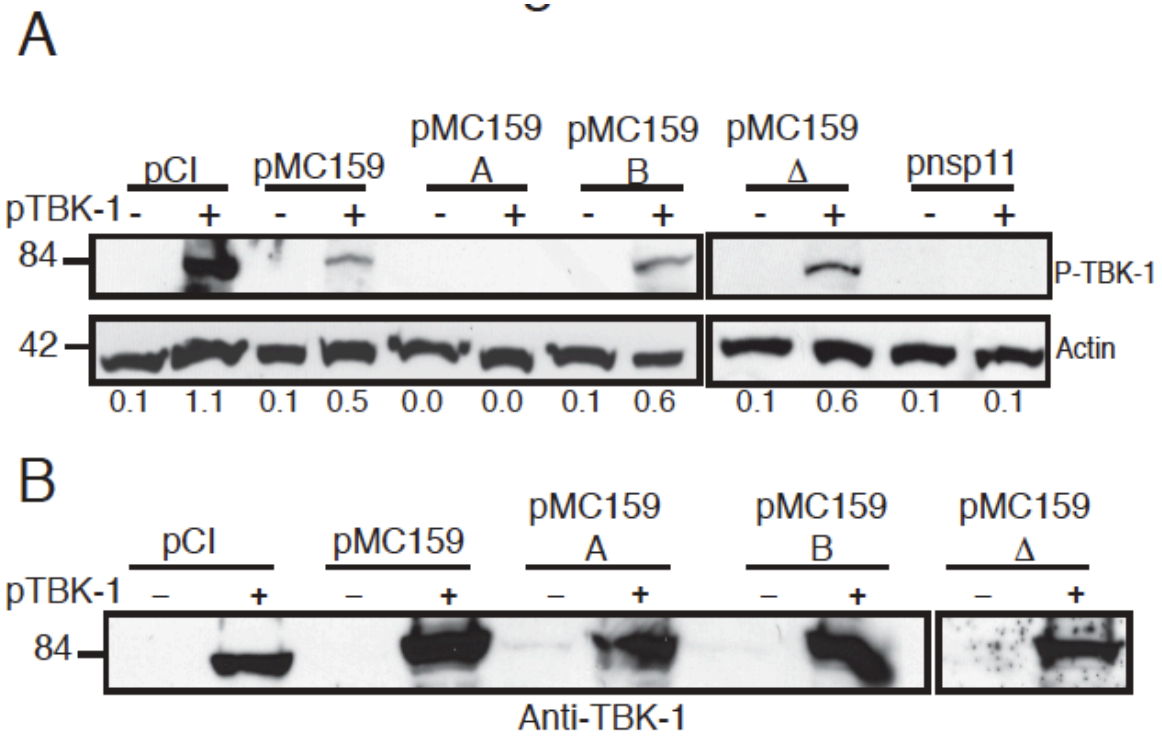

Figure 3.6 The MC159 protein inhibits TBK-1 phosphorylation. Subconfluent monolayers of 293T cells were transfected with 500 ng TBK-1 or pCI and 1000 ng pCI, pMC159, pMC159A, pMC159B, pMC159Δ or pnsp11. At 24 h post-transfection, cells were lysed and clarified cellular lysates were analyzed by immunoblot. Following SDS-10% PAGE, proteins were transferred to PVDF membranes, and were incubated with either (A) anti-phospho-TBK-1 (P-TBK-1), anti-actin antiserum or (B) anti-TBK-1 anti-serum.

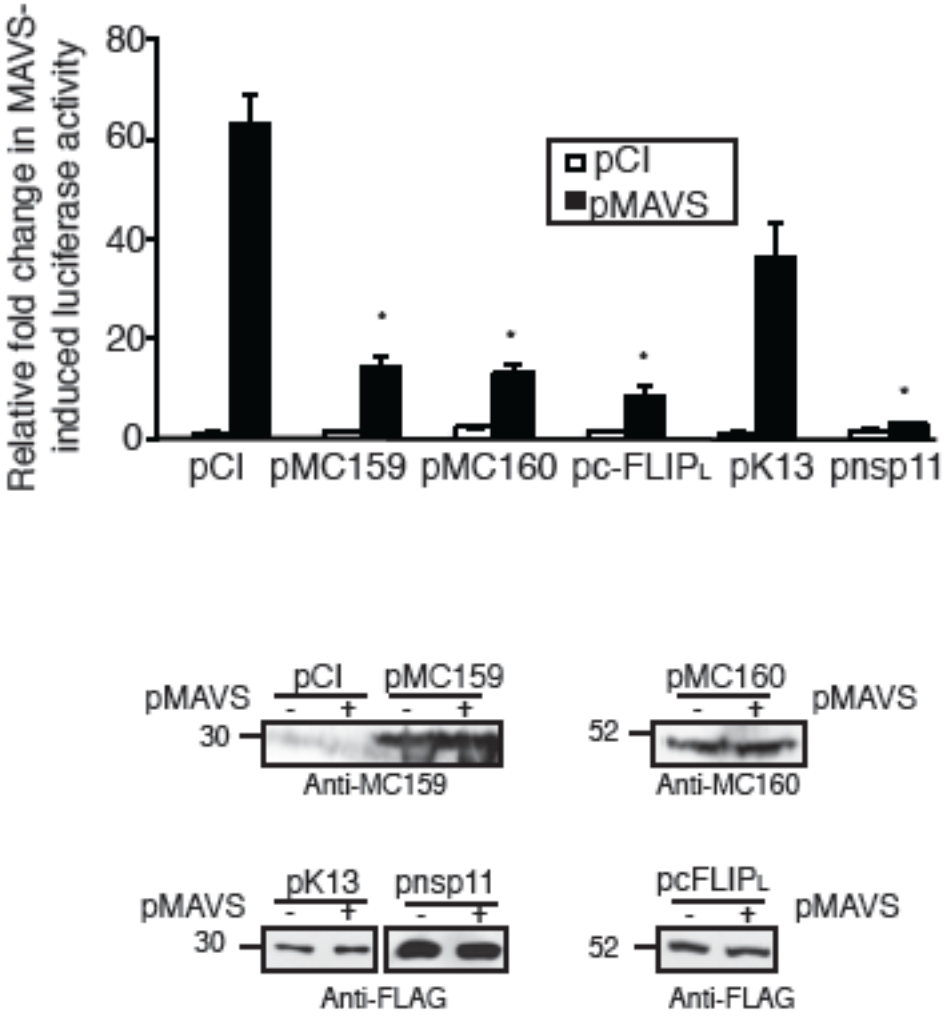

Figure 3.7 MCV MC160 and cFLIP$_L$ inhibit MAVS-induced IFN-β activation to a greater extent than KSHV K13. Subconfluent p65 -/- MEF cellular monolayers were co-transfected with 500 ng of pCI or pMAVS, 225 ng pIFN-β-luc, 25 ng pRL-null, and 1000 ng of pCI, pMC159, pMC160, pc-FLIP, pK13 or pnsp11. Transfections were performed in triplicate. After 24 h incubation, cells were lysed, and firefly and sea pansy luciferase activities were measured. Statistically significant data for FLIP inhibition of IFN-β-mediated luciferase activity ($p<0.05$) are indicated with an asterisk. A portion of each lysate was analyzed by immunoblotting. Proteins were subjected to SDS-12% PAGE, transferred to PVDF membranes and incubated with anti-MC159, anti-MC160 or anti-FLAG antiserum.

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Chapter 4: The MCV MC160 protein inhibits IFN-β expression

1) Introduction

The interferon-beta cytokine (IFN-β) is a potent activator of the anti-viral response [3]. IFN-β expression is regulated by three families of transcription factors including NF-κB, Interferon Regulator Factors (IRFs) -3 /7, and AP-1. Initiation of IFN-β expression occurs when cellular sensors called Pattern Recognition receptors (PRRs) detect of by-products of viral infection including dsRNAs [2]. Although many PRR pathways that induce IFN-β expression have different upstream pathways, most converge upon activation of the TNF receptor associated factor (TRAF3) adaptor protein. Next, TRAF3 recruits the kinase complex TANK binding kinase-1 (TBK-1): IκB kinase ε (IKKε) leading to its activation [1, 6, 11]. The TBK-1:IKKε complex phosphorylates IRF-3/7, resulting in their activation, nuclear translocation and IFN-β expression. Newly produced IFN-β is secreted, binds the interferon receptor (IFNR) on the virally infected and surrounding cells, which induces a signaling cascade resulting in the expression of interferon-stimulated genes (ISGs) [8]. Expression of ISGs produces an anti-viral state in stimulated cells.

Many viruses, including poxviruses, produce immunomodulatory proteins to combat the effects of IFN-β [7]. The molluscum contagiosum virus (MCV) encodes two immunomodulatory proteins called FLICE-like inhibitory proteins or FLIPs: MC159 and MC160 [9, 10]. FLIPs are characterized by two tandem Death Effector Domains (DEDs), which facilitate protein-protein interactions. Originally, FLIPs were characterized for their anti-apoptotic properties. Interestingly, MC160 does not inhibit apoptosis despite its

structural similarity to other FLIPs [12]. Previous studies show that MC160 is a strong inhibitor of NF-κB activation [4, 5]. The MC160 protein utilizes two mechanisms to inhibit NF-κB. First, MC160 uses regions within its DED2 to interact with caspase-8 and block IKKα activation [5]. Secondly, MC160 uses regions within its C-terminus to interact with the chaperone protein Hsp90 to destabilize the IKK complex. Together these interactions prevent degradation of the NF-κB inhibitor IκBα preventing early activation of NF-κB.

Chapter 3 showed that the MC159 protein inhibited IFN-β expression. We hypothesized that the MC160 protein may have a similar function. Here we demonstrate the MC160 protein inhibits TBK-1-induced IRF-3-driven expression of IFN-β. In addition, our data show that MC160 inhibits IFN-β using both DEDA and DEDB or its or C-terminal region.

2) Materials and methods

Cell culture and plasmids.

Human embryonic kidney 293 T cells were obtained from the American Type Culture Collection. All cells were cultured in Eagle's MEM supplemented with 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin.

The MC160/pCI plasmid contains the intact MC160 open reading frame (ORF) (Figure 4.1). The pHA-MC160 D1-D1 and pHA-MC160 D2-D2 each contain the N-terminus of the protein and either a DED1-DED1 or a DED2-DE2 in tandem, respectively. The HA-MC160 C construct consists of the C-terminus of the of the MC160

protein. The pHA-MC160 F protein contains the N-terminus and DED1 and DED2 of the MC160 protein. The pHA-MC160 D2 construct contains the second DED of MC160, and the pHA-MC160 D2C construct contains the second DED and the C-terminus of the MC160 protein. Plasmid pFLAG-TBK-1, which produces a FLAG epitope-tagged TBK-1 protein, was a kind gift from Dr. Siddharth Balachandran (Fox Chase Cancer Center). Dr. Dongwan Yoo (University of Illinois) provided the following plasmid, pMAVS, pIRF-3-luc. For all experiments involving plasmids, DNA was transfected into 293 T cells using TransIT-2020 transfection reagent (Mirusbio), following the manufacturer's protocol.

Luciferase assays.

Luciferase reporter assays were performed as previously described in chapter 3. For all luciferase assays, cellular monolayers were co-transfected with two reporter plasmids, pIRF-3-luc (225 ng) and pRL-null-luc (25 ng; Promega). For luciferase assays in which cells were transfected 500 ng pMAVS, subconfluent 293T subconfluent cellular monolayers were also co-transfected with pIRF-3-luc, pRL-null-luc, and 750 ng of either pCI, pMC160, pMC160 C, pMC159 F. At 24 h post-transfection, cells were lysed in 1X passive lysis buffer (PLB; Promega) and analyzed for firefly and sea pansy luciferase activities.

In another experiment, monolayers of subconfluent 293T cells were co-transfected with pIRF-3-luc, pRL-null-luc, and 750 ng of either pCI, pMC160, pMC160 C, pMC160 F, pMC160 D2, or pMC160 D2C. For induction of IRF-3-driven luciferase activity cells were transfected with 500 ng pTBK-1. At 48 h post-transfection, cells were

lysed in 1X PLB and analyzed for luciferase activities. In other luciferase reporter assays in which 500 ng of pTBK-1 was used, subconfluent 293T cellular monolayers were cotransfected with pIRF-3-luc, pRL-null-luc, and 750 ng of either pCI, pMC160, pMC160 D1-D1, or pMC160 D2-D2. At 48 h later, cells were lysed in 1X PLB and analyzed for luciferase activities.

All luciferase activities were measured as relative light units using a BioTek luminometer and the Dual Luciferase Reporter Assay System (Promega). All experiments were performed in triplicate. Relative luciferase activity ratios were calculated by dividing firefly luciferase activities by sea pansy luciferase activities. Luciferase activity ratios were then normalized to that of pCI-transfected cells incubated in regular medium, and this value was set as 1. Statistical significance was calculated using a Student t test where $p < 0.05$. Cellular lysates from luciferase assays were analyzed for protein expression by immunoblotting (see later).

Immunoblotting and antibodies.

The protein concentration of each clarified cellular lysate was determined using bicinchoninic acid assay (Pierce). Approximately 20 μg protein from each lysate sample was prepared for immunoblotting by re-suspending in non-reducing lane marker sample buffer (Thermo Scientific) with 5% 2-ME and boiling for 5 min.

Clarified cellular lysates were subjected to 12% SDS-PAGE, and proteins were subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore).

Membranes were incubated with indicated primary antibodies, washed three times in TBST, and incubated with appropriate HRP-conjugated secondary antibodies. Immunoblots were developed by Pierce super signal west chemilluminescence reagents.

Primary antibodies used in these experiments were polyclonal rabbit anti-MC160 (1:1000), monoclonal mouse anti-HA (1:500). Secondary antibodies conjugated to HRP were obtained from either Thermo Scientific (goat anti-mouse IgG) or Calbiochem (goat anti-rabbit IgG)

3) Results

The MC160 protein inhibits MAVS induced IRF-3-controlled luciferase activity.

The MC160 protein is a 371 amino acid protein (Figure 4.1). MC160 is comprised of two DEDs (DED 1 residues 5 to 79 and DED 2 residues 97 to 175) and a C-terminal region (residues 175 to 371) that lacks homology to any known proteins. A series of MC160 mutants from previous studies was used to determine the NF-κB inhibitory regions of MC160 (Figure 4.1) [5].

To characterize MC160-mediated inhibition of IFN-β expression, we performed dual luciferase reporter assays in which the luciferase reporter plasmid was under the transcriptional control of the IRF-3 promoter. Here we tested the following MC160 constructs: wild-type (WT) MC160, MC160 C (containing only the C-terminus) and MC160 F (containing only DED1 and DED 2) for the ability to inhibit MAVS-induced IRF-3 activation (Figures 4.1, 4.2, Table 4.1). Interestingly, both MC160 (WT) and MC160 F inhibited IRF-3-driven luciferase activity 5-fold over vector-transfected cells,

indicating that MC160 indeed inhibited IRF-3 activation. Moreover, the DED1 and DED2 regions of MC160 were sufficient for this inhibition. However, MC160 C did not inhibit IRF-3-driven luciferase activity, suggesting that its C-terminus is dispensable for inhibition. MC160 F protein levels were comparable to MC160 and MC160 C protein levels, suggesting that its lack of function was not due to low protein levels. From these data, we concluded that MC160 utilizes regions with in its DEDs to inhibit IFN-β expression.

The MC160 protein inhibits TBK-1 induced IRF-3-controlled luciferase activity.

Next we assessed MC160's ability to inhibit IRF-3 driven luciferase activity downstream of the TANK Binding Kinase -1 (TBK-1) (Figure 4.3, Table 4.1) [2]. TBK-1 is part of a kinase complex that is essential for IRF-3 driven expression of IFN-β. Since the MC159 protein inhibited TBK-1 activation, (Chapter 3), we hypothesized that the homologous MC160 would have a similar function. Similar to Figure 4.2, WT MC160 and MC160 F inhibited IRF-3-driven luciferase activity (Figure 4.3). However, MC160 D2, which only contains the DED2 of MC160, did not inhibit luciferase activity, implying that the DED1 of MC160 is important for inhibition. Interestingly, MC160 C, which did not inhibit MAVS-induced IRF-3 activation, inhibited TBK-1-induced IRF-3-driven luciferase activity (Figures 4.2, 4.3). Finally, MC160 D2C, which contains the DED2 and the C-terminus of MC160, also significantly inhibited luciferase activity.

The DED 2 of the MC160 protein is required for inhibition of MAVS induced IRF-3-controlled luciferase activity.

To further elucidate the role of the DEDs in MC160 mediated inhibition of IFN-β expression we test two MC160 constructs, in which either DED1 or DED2 were repeated in tandem (MC160 D1-D1 and MC160 D2-D2, respectively) (Table 4.1). MC160 chimeras were assayed for their ability to inhibit MAVS-induced IRF-3 activation (Figure 4.4). The MC160 wild-type and MC160 DED2-DED2 significantly inhibited IRF-3-driven luciferase activity over 4-fold, suggesting DED2 is required for inhibition. Conversely, MC160 D1-D1 did not inhibit.

4) Discussion

Here the MCV MC160 protein is described as an inhibitor of anti-viral response. We show that MC160 inhibits IFN-β expression by blocking IRF-3-controlled luciferase activity. Therefore MC160 mediated inhibition of IFN-β expression is independent of other IFN-β regulators such as, NF-κB. This suggests MC160 uses different mechanism for inhibition of NF-κB activation and IFN-β expression.

Our results indicate that the DEDs of MC160 are sufficient for inhibition of MAVS- and TBK-1-induced IRF-3-driven luciferase activity (Figures 4.2, 4.3). However, an MC160 chimera that expresses two tandem DED1s no longer inhibits MAVS-induced IRF-3 activation (Figure 4.4). Conversely, a chimera that expresses two tandem DED2s strongly inhibits MAVS-induced-IRF-3 activation, suggesting DED2 is required for inhibition. However a single DED2 does not inhibit IRF-3 activation (Figure 4.3). Our data indicate that DED2 is required but not sufficient for inhibition of IRF-3-driven IFN-

β expression (Table 4.1). We hypothesize that the reason for this difference is DED2 mediates a specific interaction for inhibition but two DEDs are required to maintain the proper structure for these interactions.

We observed that the C-terminus of MC160 did not inhibit MAVS-induced IRF-3 activation, but did inhibit TBK-1 induced IRF-3 activation (Figures 4.2, 4.3). It is unclear why the phenotype of the MC160 C mutant is different for these inducers. One possibility is that the C-terminus targets are part of the pathway that is not required for MAVS-induced IRF-3-driven transcription. A second possibility is the observed differences are an artifact of the assay perhaps from over-expression. Regardless, inhibition of IRF-3-driven transcription by MC160 F and MC160 C suggests that MC160 may utilize two mechanisms for inhibition. It should be noted that MC160 inhibits NF-κB using two distinct mechanisms; the C-terminal region of MC160 binds to Hsp90 to ultimately de-stabilize IKK complexes, while the MC160 DED2 binds to procaspase-8 to inhibit capase-8-induced NF-κB activation. Thus, it would not be unreasonable to think that the MC160 C-terminus and DEDs inhibit IFN-β expression through distinct mechanisms. Recently, Hsp90 was shown to be necessary for IRF-3 activation [13]. To this end, we hypothesize that MC160C:Hsp90 interactions may be responsible for the inhibition of IFN-β expression.

Additional studies are needed to fully understand the molecular mechanisms of MC160 mediated inhibition of IFN-β expression, and future directions include studying the effect of these mutant MC160 proteins on TBK-1 phosphorylation and IRF-3 activation.

5) Figures

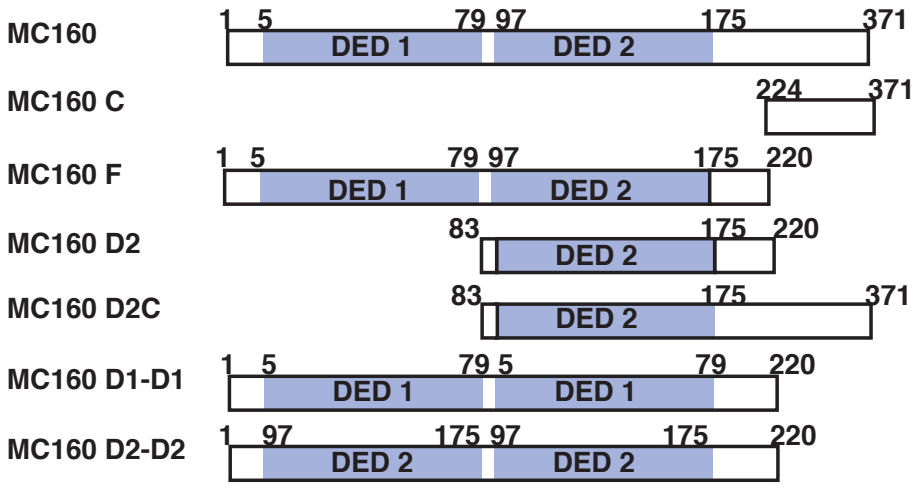

Figure 4.1 MC160 WT and mutant protein constructs (adapted from Nichols, 2009). Diagram of WT MC160 protein 371- amino-acids in length with two DEDs; DED1 (residues 5-79) DED2 (residues 97-175) and C-terminal region (residues 175 to 371). Below the WT MC160 protein are MC160 mutants C, F, D2, D2C, D1-D1 and D2-D2. An HA epitope tag was introduced at the N-terminus of each mutant protein construct.

MC160 Construct	NF-κB	MAVS-IRF-3	TBK-1-IRF-3
MC160 F	+	+	+
MC160 C	+	-	+
MC160 D2	+	NA	-
MC160 D2C	+	NA	+
MC160 D1-D1	NA	-	NA
MC160 D2-D2	NA	+	NA

Table 4.1 Summary of MC160 protein phenotypes for inhibition of NF-κB, MAVS-induced IRF-3 activation and TBK-1-induced IRF-3 activation. (+) Indicates inhibition, (–) indicates loss of inhibition (NA) data not available.

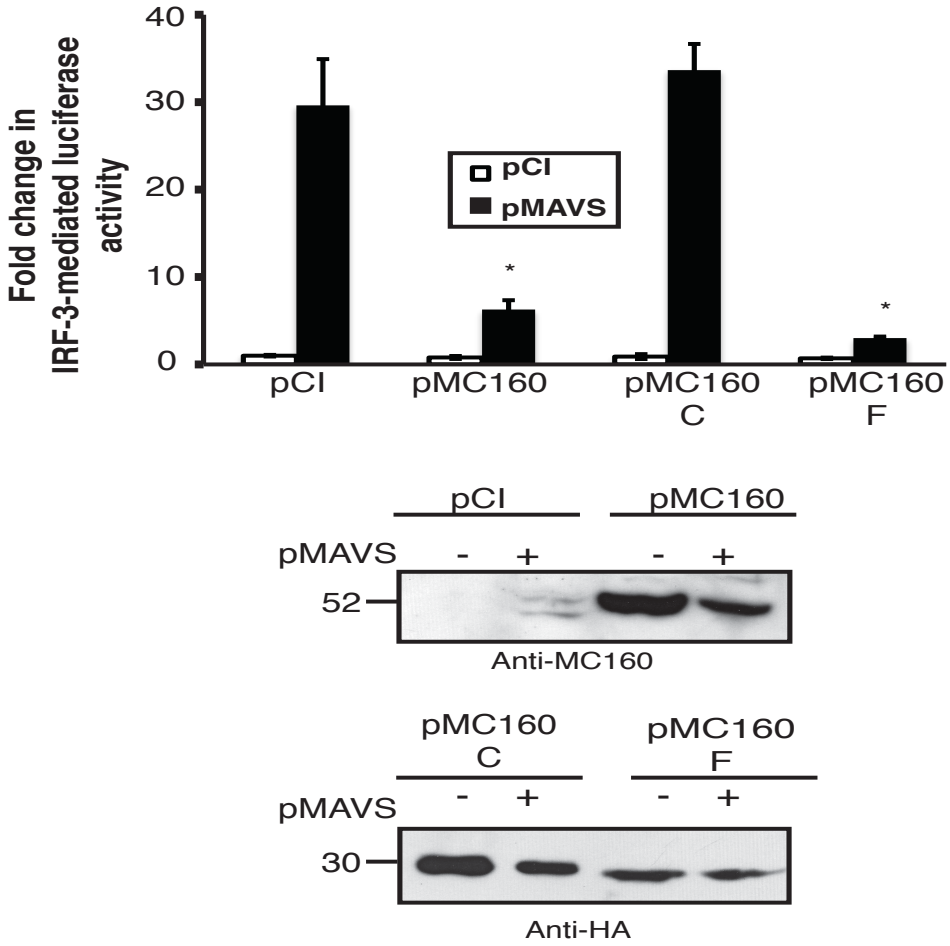

Figure 4.2 The MC160 protein inhibits MAVS induced IRF-3-controlled luciferase activity. Subconfluent 293T cellular monolayers were transfected with pIRF3-luc (225 ng), pRL-null-luc (25 ng), and 1000 ng of pCI, pMC169, pHA-MC160 C, pHA-MC160 F. For activation of IRF-3, cells were also transfected with 500 ng of pCI of pMAVS. After 24 h incubation, cells were lysed and luciferase activities were measured. Transfections were performed in triplicate. Statistically significant data for MC160 inhibition of IRF3-mediated luciferase activity ($p<0.05$) are indicated with an asterisk. Lysates were analyzed by immunoblotting. Proteins were subjected to SDS-12% PAGE, transferred to PVDF membranes and incubated with an anti-MC160 or anti-HA antiserum.

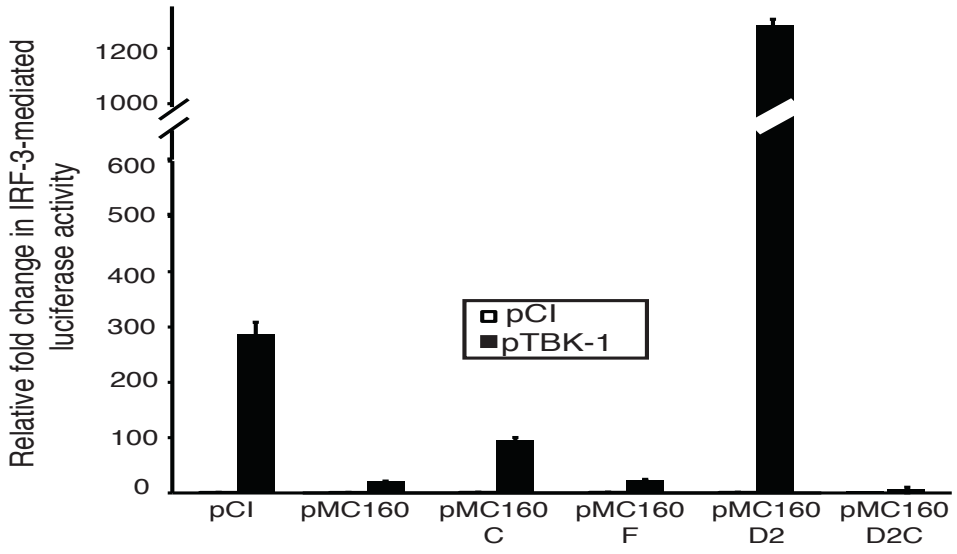

Figure 4.3 The MC160 protein inhibits TBK-1 induced IRF-3-controlled luciferase activity. Subconfluent 293T cellular monolayers were transfected with pIRF3-luc (225 ng), pRL-null-luc (25 ng), and 1000 ng of pCI, pMC160, pMC160 C, pMC160 F, pMC160 D2 or pMC160 D2C. For activation of IRF-3, cells were also transfected with 500 ng of pCI of pTBK-1. After 24 h incubation, cells were lysed and luciferase activities were measured. Transfections were performed in triplicate. Statistically significant data for MC159 inhibition of IRF3-mediated luciferase activity ($p<0.05$) are indicated with an asterisk.

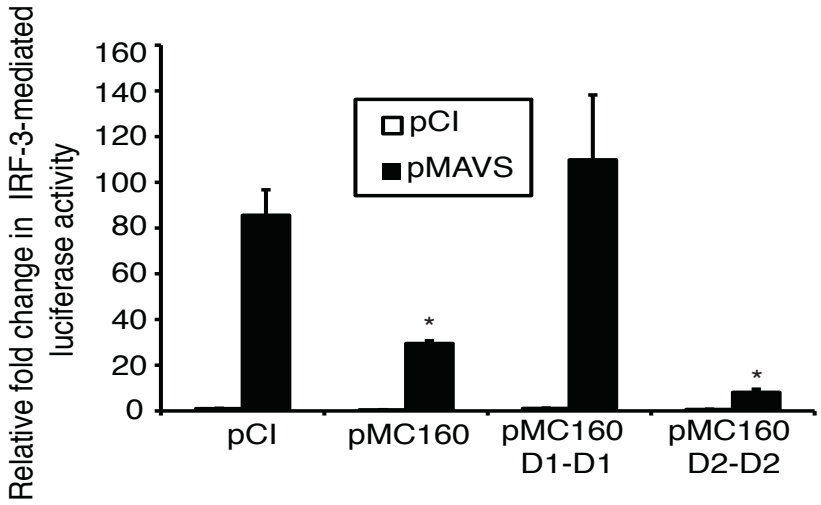

Figure 4.4 The MC160 protein inhibits MAVS induced IRF-3-controlled luciferase activity. Subconfluent 293T cellular monolayers were transfected with pIRF3-luc (225 ng), pRL-null-luc (25 ng), and 1000 ng of pCI, pMC160, pMC160 D1-D1, pMC160 D2-D2. For activation of IRF-3, cells were also transfected with 500 ng of pCI of pMAVS. After 24 h incubation, cells were lysed and luciferase activities were measured. Transfections were performed in triplicate. Statistically significant data for MC159 inhibition of IRF3-mediated luciferase activity ($p<0.05$) are indicated with an asterisk.

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Chapter 5: Summary of thesis and future directions

1) Summary

The MC159 protein is a potent inhibitor of anti-viral responses. Previous studies showed that MC159 could inhibit apoptosis and NF-κB activation [3, 4, 6]. My work elucidated the molecular mechanism of MC159 mediated inhibition of NF-κB [9]. Additionally, I describe a new function for the MC159 and other FLIPs in inhibition of IFN-β expression.

Originally MC159 was thought to target the TRAF2 adaptor molecule to inhibit NF-κB [6]. I found MC159 can inhibit NF-κB activation downstream of TNF, PMA and MyD88 (Chapter 2). Since PMA induction of NF-κB expression is TRAF2 independent, I hypothesized that MC159 inhibits NF-κB an event downstream of TRAF2 recruitment. I found that MC159 co-associates with the IKKγ subunit of the IKK complex. Previous studies in our lab showed MC159 prevents activation of the IKKβ subunit of this complex. Together these data suggest MC159 interacts with the IKK complex to block activation of NF-κB. Mutational analysis of the MC159 protein revealed that the RxDL motif within the N-terminal DED of MC159 is required for inhibition.

Recent research suggests that MC159 may modulate IFN-β expression through interactions with the apoptotic and signaling molecule FADD [1]. However, these studies did not evaluate if this inhibition was independent of NF-κB. This thesis details several lines of evidence that suggest MC159 mediated inhibition of IFN-β expression is NF-κB independent (Chapter 3). First, I found MC159 inhibits IFN-β promoter controlled

transcription in NF-κB deficient cells. Secondly, my data show that IRF-3 and TBK-1, essential regulators of IFN-β expression, are inactive in MC159 expressing cells. Furthermore, I show that neither DED of MC159 is required for inhibition of IFN-β expression. Finally, I evaluated other FLIPs ability to inhibit IFN-β expression. Here I show MC160 and $cFLIP_L$ inhibit IFN-β expression in NF-κB deficient cells. Interestingly, FLIP K13 did not inhibit IFN-β expression.

To further evaluate the mechanism of FLIP mediated inhibition of IFN-β, I characterized a series of MC160 deletion mutants (Chapter 4). Similar to MC159, MC160 is a potent inhibitor of NF-κB activation. I found MC160 inhibits IRF-3-driven transcription to inhibit IFN-β expressing, suggesting MC160 also acts independently of NF-κB.

2) Future directions

MC159 inhibition of NF-κB.

As a result of my thesis work, it is clear that MC159 co-associates with the IKK complex to block NF-κB activation (Chapter 2). However, it is still unclear how these interactions occur. Further research is needed to understand how MC159 co-associates with the IKKγ subunit of the IKK complex, and to determine whether they interact directly or via an intermediate. If MC159 and IKKγ directly interact, more studies are needed to determine the regions of MC159 and IKKγ that mediate this interaction. Since IKKγ is regulated by multiple ubiquitination and phosphorylation events, we hypothesize that MC159 prevents IKKγ modifications, thus preventing it from staying in an active

confirmation [5, 10]. Therefore, future efforts will focus on evaluating IKKγ modifications in MC159 expressing cells.

MC159 inhibition of IFN-β.

This thesis describes a new function for the MC159 protein in inhibiting IFN-β expression. I found that either DED of MC159 is sufficient to inhibit IFN-β, but neither DED is required (Chapter 3). Therefore, it remains unclear what regions facilitate this inhibition. Further mutational analysis of MC159 is needed to identify the regions required for this function. My data show that MC159 can block TBK-1 phosphorylation suggesting it may interact with the TBK-1 kinase complex. However, the molecular mechanism of inhibition remains unclear. Future efforts will focus on determining if MC159 interacts of TBK-1, IKKε or other signaling molecules. Finally, my data show that MC159 specifically prevents IRF-3 phosphorylation and in turn IFN-β production. Currently it is unclear if MC159 inhibits the activity of other IFN-β regulators, such as IRF-7. Like IRF-3, IRF-7 is activated by TBK-1; therefore I predict that IRF-7 would also be inhibited in the presence of MC159 [12, 13]. Since IRF-7 also regulates IFN-α expression, more studies are needed to determine if MC159 modulates IFN-α [8].

MCV pathogenesis.

Although much is known regarding how the MC159 protein functions *in vitro*, little is known concerning how MC159 contributes to MCV pathogenesis. Unfortunately there is no tissue culture or animal model for MCV [2]. However, it may be possible to express MC159 in a surrogate virus to study its function during infection. This method

presents significant challenges because many poxviruses encode myriad immune evasion proteins whose functions overlap with MC159 [11]. Currently, ORFV, a poxvirus that infects sheep, is the top candidate for a surrogate virus for studying MCV immune evasion molecules because of its tropism for the skin [15]. Future efforts will focus on developing a viable model system to study effects of MCV immune evasion proteins on pathogenesis.

FLIP functions.

In chapter 3, I describe two additional FLIPs that inhibit IFN-β expression, MC160 and $cFLIP_L$. However, the molecular mechanisms of inhibition remain unclear for both proteins. Future studies will focus on determining the functional regions of these proteins and identifying binding partners that facilitate IFN-β inhibition.

Preliminary mutational analysis of MC160 revealed that both the DEDs and C-terminus of MC160 are sufficient for inhibition (Chapter 4). Further studies are needed to determine the molecular mechanism of inhibition for MC160 DEDs and MC160 C-terminus. Interestingly, the MC160 C-terminus is known to interact with Hsp90 to inhibit NF-κB activation [7]. Recently, Hsp90 was shown to play a role in activation of IFN-β expression by interacting with TBK-1:IKKε complex [14]. We hypothesize that MC160 C is interacting with Hsp90 to block IRF-3-driven IFN-β expression. Therefore, future studies will evaluate the role of Hsp90 in MC160 mediated inhibition of IFN-β expression.

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